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The Journal of Laboratory and Clinical Medicine

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CLINICAL AND EXPERIMENTAL

BLOOD AMMONIA AND ELECTROLYTES IN HEPATIC COMA

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ALTHOUGH biochemical abnormalities have been observed in patients with hepatic coma, their specific etiologic importance has not been demonstrated. An abnormality in ammonia metabolism has been suggested by the observations of previous investigators who noted elevation of the blood ammonia in patients with cirrhosis of the liver, a few of whom also had hepatic coma.[1-4] In addition, the administration of certain nitrogenous substances to patients with cirrhosis of the liver sometimes produces a clinical syndrome indistinguishable from impending hepatic coma.[5] Previous studies have indicated a rising blood pyruvate level in patients with hepatic coma, particularly after glucose administration.[6, 7] More recently, Amatuzio and his associates[8] have reported a decrease in the concentration of serum cations and of phosphorus in similar patients.

The present study was undertaken to define the blood acid-base, electrolyte, and ammonia patterns encountered in patients with hepatic coma and to determine the existence of any relationship between chemical and clinical observations.

MATERIALS AND METHODS

The observations reported herein were made on some of the patients with liver disease who were on the wards of the Boston City Hospital between October, 1951, and July, 1952. Twenty-two patients, of whom fifteen were men, were studied. All were chronic alcoholics

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass., and the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Md.

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*On assignment from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

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TABLE I. CLINICAL AND LABORATORY DATA IN PATIENTS WITH CIRRHOSIS OF THE LIVER WITH COMA

PATIENT	AGE	SEX	DATES ADM.	DATES DIED	ASCITES 0-4+	EDEMA 0-4+	LIVER SIZE CM.	BSP RETENTION IN 45′ %	I. I. UNITS	TREMOR	MENTAL STATUS	EEG DATE
Group I. *Impending Coma*												
J. M.	61	M	10/18/51	11/14/51*	2+	2+	Enlarged	51.8 (8/4/51)	20.0	Definite	Mildly Confused	Characteristic† 10/10/51 (OPD)
L. F.	40	M	11/17/51	3/4/52*	1+	0	7.0	22.5	—	Definite	Confused Disoriented	Abnormal 11/27 Normal 12/11-12/17
J. O'B.	42	M	4/8/52	8/29/52*	4+	3+	8.0	52.0	—	Slight	Confused	—
J. S.	42	M	5/13/52	7/1/52*	0	0	10.0	54.0	—	Definite	Oriented Sluggish	—
J. L.	59	M	12/17/51	12/27/51	2+	0	?	36.2	—	Definite	Disoriented	—
C. J.	62	M	2/14/52	2/28/52	2+	1+	6.0	48.2	—	Definite	Confused	—
J. P.	77	M	3/8/52	3/12/52	4+	0	N.P.	—	12.5	Definite	Lethargic	Abnormal 2/20/52
A. R.	40	F	1/28/52	2/25/52	2+	1+	12.0	34.0	100.0	Definite	Oriented	Characteristic 2/21/52
C. R.	55	M	10/17/51	10/23/51	2+	0	Enlarged	44.0	—	Definite	Semi-coma	—
J. C.	58	M	5/29/52	6/8/52	2+	0	10.0	66.0	100.0	Slight	Disoriented Semi-coma	—
A. A.	68	M	4/11/52	4/17/52	2+	0	5.0	53.5 (3/21/52)	—	Slight	Disoriented	—
L. S.	31	F	11/12/51	11/14/51	0	0	4.0	46.0	—	Definite	Disoriented Lethargic	Characteristic 11/13/51
E. B.	49	F	10/30/51	11/16/51	2+	2+	—	—	40.0	Definite	Lethargic Disoriented	Characteristic 11/14/51
A. McL.	51	F	6/26/52	7/1/52	2+	1+	—	45.4	—	Definite	Disoriented	—
J. B.	68	M	5/5/52	5/27/52	4+	4+	—	61.2	—	Slight	Confused Disoriented	—

						Group II. *Coma*					
M. McL.	43	M	9/29/51 10/4/51	3+	0	7.0	40.0 (1948)	—	None	Deep Coma	—
J. M.	62	M	2/14/52 2/17/52	2+	1+	7.5	51.9 (8/4/51)	—	Slight	Deep Coma	—
B. W.	51	F	10/27/51 11/10/51	2+	0	7.0	—	100.0	None	Deep Coma	Characteristic 10/31/51
A. DeG.	67	M	11/4/51 11/7/51	3+	1+	N.P.	44.0 (6/27/51)	—	None	Deep Coma	—
J. H.	42	F	2/17/52 3/3/52	2+	1+	N.P.	80.0	100.0	None	Deep Coma	—
M. O'B.	38	F	3/23/52 3/24/52	+ –	0	4.0	24.0 (1948)	50-75	None	Deep Coma	—
T. S.	71	M	6/5/52 6/13/52	2+	0	6.0	—	60.0	None	Stuporous	—
T. Sc.	44	M	5/26/52 6/11/52	1+	1+	8.0	—	70.0	Definite	Coma	—

*Patient recovered from the neurological complications and was discharged from the hospital on the date given.

†The characteristic electroencephalographic findings consist of bilaterally synchronous slow waves of two-per-second frequency, occurring in bursts separated by cortical alpha rhythm. Initially only the frontal areas are involved, but with progression the entire record may show continuous slow wave activity.

TABLE II. BIOCHEMICAL OBSERVATIONS

PATIENT	DATE OF SAMPLE	SERUM					BLOOD			
		$pH_{38°}$	$(CO_2)_s$ (mM/L.)	Cl (mM/L.)	Na (mM/L.)	K (mM/L.)	NH_3-N (μG/ML.)	NPN (MG. %)	HCT. (%)	Hb (GM. %)
Group I—Impending Coma										
J. M.	10/10/51	7.47	26.3	105.8	129.2	3.4	10.0	27.6	31.2	10.4
L. F.	11/26/51	7.42	25.9	99.2	138.9	4.0	4.6	——	33.5	10.8
J. O'B.	4/ 8/52	7.45	24.3	110.0	140.5	3.6	2.8	46.0	30.0	9.9
J. S.	5/16/52	7.30	29.9	92.3	136.1	3.0	5.5	31.0	42.8	——
J. L.	12/19/51	7.38	25.4	101.6	141.5	5.1	2.0	——	30.0	9.7
C. J.	2/14/52	——	31.0	107.1	140.0	3.5	3.0	——	35.2	10.0
J. P.	2/14/52	7.45	——	108.5	139.0	4.2	3.4	——	——	——
A. R.	2/19/52	7.32	18.6	85.9	119.0	2.5	4.4	——	32.0	10.3
C. R.	10/22/51	7.50	28.1	106.9	147.2	2.8	6.8	——	47.0	15.1
J. C.	6/ 5/52	7.40	23.3	85.8	117.5	3.2	2.1	44.4	36.0	——
A. A.	4/14/52	7.42	15.4	110.2	131.2	4.5	4.4	82.5	39.5	13.0
L. S.	11/13/51	7.38	36.3	——	127.5	3.6	—	——	22.0	6.7
E. B.	11/14/51	7.30	20.6	78.2	120.0	5.9	4.0	——	35.0	11.0
A. McL.	6/28/51	——	——	116.1	144.8	3.8	—	——	——	——
J. B.	5/ 8/52	7.48	22.5	105.9	135.0	3.3	3.2	30.0	27.0	9.2
Mean		7.41	25.2	101.0	133.8	3.76	4.32	43.6	33.9	10.6
Group II—Coma										
M. McL.	10/ 1/51	——	28.8	——	109.0	2.8	12.3	——	——	——
J. M.	2/15/52	7.52	21.6	100.8	138.5	4.0	6.8	——	27.0	9.0
B. W.	11/ 3/51	7.50	25.6	95.9	133.1	2.8	4.6	——	——	——
A. DeG.	11/ 5/51	7.50	21.8	111.0	127.0	5.0	8.4	——	——	——
J. H.	2/27/52	7.50	26.0	77.0	108.1	2.6	3.2	22.0	35.2	10.2
M. O'B.	3/24/52	7.42	24.0	——	140.2	3.5	2.0	41.0	41.0	14.0
T. S.	6/10/52	7.40	17.3	100.7	132.5	4.2	2.0	54.0	31.0	10.1
T. Sc.	6/11/52	7.35	25.8	88.2	122.0	3.8	15.4	47.0	39.6	——
E. B.	11/16/51	7.23	12.0	86.2	120.0	—	7.2	——	34.5	——
J. L.	12/26/51	7.38	17.3	110.3	138.2	7.0	—	——	13.5	——
C. J.	2/27/52	7.42	20.0	103.7	135.5	3.9	2.6	165.0	33.2	9.7
J. B.	5/26/52	7.40	20.9	102.3	134.0	5.2	4.5	76.0	35.5	11.0
A. McL.	6/30/52	7.52	27.4	112.5	137.0	3.1	3.6	48.0	43.0	13.0
Mean		7.43	22.2	98.6	128.8	4.0	6.0	64.7	33.4	11.0
Normal Range		7.35-7.48	25-28	104-109	137-146	3.5-5.0	1.0-3.0	25-40	——	——

with advanced cirrhosis of the liver. This diagnosis was confirmed by pathologic examination in ten patients; one other had coincident cirrhosis and hepatoma. The identifying data are given in Table I. The patients have been arbitrarily classified clinically into two groups: (1) *Impending coma:* those patients with a clouded sensorium, confusion, disorientation, and a coarse "flapping" tremor (a flexion-extension movement at the metacarpophalangeal and wrist joints best elicited by active posturing of the outstretched hand, and when more severe demonstrable in other skeletal muscle groups).[9] (2) *Coma:* those patients unresponsive to painful stimuli, and with occasionally absent corneal reflexes. The flapping tremor occasionally could be elicited in comatose patients unable to cooperate by passively applying tension to muscle groups, for example, by firmly flexing the wrist. The patients are classified in Table I according to their status at the time of the initial observations, although serial observations were usually made. Whenever possible, electroencephalograms were obtained coincident with clinical and chemical observations.*

The data given in Table II were obtained by the following chemical analytical techniques: for serum, the $pH_{38°}$ by the method of Hastings and Sendroy,[10] the carbon dioxide content as described by Van Slyke and Neill,[11] the chloride concentration by the iodometric titration of Van Slyke and Hiller,[12] and the sodium and potassium concentrations by internal standard flame photometry[13]; for blood, nonprotein nitrogen concentration by the method of Folin[14] and ammonia content by a modification of the microdiffusion technique of Conway and Cooke.[15]†

RESULTS

The ominous prognosis of liver coma is indicated by the death in coma of twenty of the twenty-two patients. When first observed, eleven of these patients showed signs of impending coma, while seven were comatose. Four other patients (J. M., L. F., J. O'B., and J. S.) with impending coma had transient symptoms. Of the latter, two (L. F. and J. O'B.) have remained well. The other two died within six months of the onset of symptoms, although there was an interval of several months during which the patients were free of symptoms. The average duration of hospitalization of the patients with impending coma with a progressive clinical course was eleven days, with a range from two to twenty-eight days. The duration of hospitalization prior to death of the patients initially observed in coma averaged eight days, with a range from one to sixteen days. Of the entire group only two patients did not have ascites and edema. Factors possibly contributing to the onset of coma included the administration of opiates, especially as paregoric (patients A. R. and M. McL.), and ammonium chloride as a diuretic (patients J. L. and T. S.), as well as paracentesis (J. B. and A. DeG.), transfusion reaction (T. S.), and hematemesis (J. L., A. McL., and M. O'B.). Electroencephalographic tracings were obtained from seven patients; five of these showed delta wave activity characteristic of impending hepatic coma, as described by Foley, Watson, and Adams.[16]

The findings presented in Table II indicate the marked variability in individual biochemical patterns. The mean values for serum $pH_{38°}$, carbon dioxide

*The Neurological Unit of the Boston City Hospital provided the facilities for these observations, and Dr. Joseph Foley interpreted the records.

†The blood for the determination of ammonia was collected with minimal venous stasis into an oiled syringe and was transferred into a flask containing potassium oxalate crystals and filled with 100 per cent carbon dioxide. Diffusion from 1 ml. samples in Conway units was allowed for sixty minutes. No corrections were made for the differences in the diffusion rates of ammonia between ammonium sulfate standard and blood or for the deaminating action of the alkali. The data thus obtained represent free ammonia as well as nitrogenous substances in blood capable of releasing ammonia under the above conditions. The data are expressed as μg ammonia nitrogen per milliliter whole blood.

content, potassium concentration, and hematocrit or hemoglobin concentration, do not differ in the two groups of patients. However, three points are noteworthy: (1) the mean blood ammonia is elevated in both groups, (2) the mean serum sodium and chloride* concentrations show a progressive decrease in the two groups, and (3) the mean blood nonprotein nitrogen is elevated in the coma group.

Blood ammonia determinations in thirteen normal individuals indicated a mean value of 2.04 (standard deviation 0.45), while similar determinations on twelve patients with cirrhosis without neurologic complications indicated a mean of 2.74 (standard deviation 0.86) micrograms N per milliliter of blood. The patients in the two groups with neurologic findings showed significant elevation of the mean blood ammonia concentrations, although there was marked variability of individual values within the groups from normal to highly elevated values. Neither the presence of a "flapping" tremor nor the state of consciousness correlated well with the blood ammonia values in serial observations.

An initial uncompensated metabolic acidosis was noted in only two patients with impending coma and in one with coma. Although hyponatremia was generally present, as indicated by the mean values in both groups, markedly diminished serum sodium concentrations (120 mM per liter or less) occurred in three patients in each group. In only one instance was an elevated serum concentration of sodium observed. Three patients in each group were also hypokaliemic (3.0 mM per liter or less). The one instance of hyperkaliemia in the coma group might be attributed to hematemesis and repeated blood transfusions.

Although very low levels of serum sodium and potassium concentrations were noted in several patients, no relationship between hyponatremia and/or hypokaliemia and neurologic status was noted. Moreover, neither was there any such relationship evident in serial measurements of these electrolytes in individual patients. The following case is illustrative (see Table III).

TABLE III. SERIAL MEASUREMENTS OF PATIENT A. D.; IMPENDING COMA

DATE	$pH_{38°}$	(CO_2)	Cl	Na	K	$(NH_3-N)_p$	NPN	HCT.	CLINICAL STATUS
		mM/L. SERUM				μG/ML.	(MG. %)	(%)	
1/5/53	7.48	26.8	89.1	122.1	3.8	1.4	25.6	40.0	Impending coma
1/6	—	—	82.5	117.5	3.4	—	23.6	—	Impending coma
1/7*	7.42	27.4	83.5	120.0	3.8	1.6	24.4	38.0	Impending coma
1/8 9 A.M.	7.45	26.5	81.1	119.3	4.0	1.8	—	34.8	Impending coma
1 P.M.†	—	—	83.6	118.0	4.2	—	—	—	Impending coma
5 P.M.	—	—	100.0	133.5	4.5	—	—	—	Impending coma
1/9 12 noon	7.42	26.1	89.8	124.4	4.4	1.9	—	35.0	Impending coma
1/12	—	—	95.1	131.0	—	—	—	—	Coma
1/13	7.50	30.5	90.4	126.8	3.5	2.6	29.2	40.0	Coma
1/15	—	—	—	128.0	3.2	—	—	—	Coma

*Patient received 250 ml. of 25 per cent dextrose intravenously.

†Patient received 500 ml. of 5 per cent sodium chloride intravenously during four hours. No fluids were given during the subsequent twenty-four hours.

*The serum chloride concentration in general paralleled that of serum sodium and is omitted from further discussion.

Patient A.D., a 42-year-old white woman, a chronic alcoholic, was admitted to the hospital because of the recent onset of ascites and jaundice. Physical examination revealed a temperature of 97.8° F. p.o., pulse, 110; respirations, 32; blood pressure 110/70, weight 62.2 kg. Spider telangiectasia, palmar erythema, marked ascites, and moderate ankle edema were present. There was evidence of recent weight loss. Although the patient was oriented, she was slow in response, and had a "far away" look. There was no confusion, nor could a "flapping" tremor be demonstrated. Initial chemistries, obtained on admission, indicated a serum sodium concentration of 122.1 mM per liter, normal pH, carbon dioxide content, serum potassium concentration, and blood nonprotein nitrogen content. Within twenty-four hours, confusion, disorientation, and a flapping tremor were noted. These persisted unchanged for five days, although alterations in serum electrolytes occurred. On the third day, fluid was restricted and 250 c.c. of 25 per cent glucose was administered intravenously, but no effect on the clinical status was discernable. On the fourth day, because the serum sodium concentration was 119.3 mM per liter, hypertonic sodium chloride (500 c.c. of 5 per cent solution) was administered and fluid restricted for twenty-four hours without effect on clinical status, although a significant rise in the serum concentrations of sodium and chloride was obtained transiently. Thereafter minimal fluids with concentrated glucose solutions were given and excessive hyponatremia avoided. Subsequently deep coma ensued following a hematemesis of approximately 300 c.c. The patient was maintained on blood transfusions and parenteral glucose but succumbed ten days after admission.

Although blood was not routinely analyzed for nonprotein nitrogen, the data obtained is presented to indicate that azotemia was not commonly noted in initial observations. In serial measurements several patients were observed to develop hepatic coma without azotemia; whether this absence of azotemia represents adequacy of renal excretion of urea or failure of synthesis in the liver cannot be stated. In contrast, the significant elevation of the blood non-protein-nitrogen noted in four patients, indicated failure of renal excretion since elevation of the non-protein-nitrogen constituents of the blood other than urea could not explain these values even in complete liver failure.

DISCUSSION

The role of ammonia in the development of hepatic coma has not been clearly established. Burchi,[1] Fuld,[2] and Kirk[3] previously noted elevated blood ammonia values in patients with cirrhosis. In addition to these authors, Van Caulaert and Deviller[17] found significant elevations of the blood ammonia content following the oral administration of ammonium salts to patients with liver disease. Traeger and associates[4] verified these findings recently and also observed that the highest values occurred in patients with hepatic coma. The present observations confirm this finding. However, the marked variation in individual values as well as the failure to show a direct correlation between blood ammonia content and the clinical state may well indicate concomitant changes rather than cause and effect.

Although the mean data from patients with hepatic coma indicate normal acid-base equilibrium and electrolyte concentrations except for hypochloremia and hyponatremia, the data varied greatly from patient to patient. Thus, although individual abnormalities occurred in all measured parameters, no consistent biochemical pattern was observed. In particular, neither acidosis nor hypokaliemia were outstanding findings and cannot be considered seriously

as important causative factors in the development of hepatic coma. These observations are consistent with those reported in a previous study in which the syndrome of impending hepatic coma was produced in patients with cirrhosis of the liver without significant alterations in serum pH or electrolyte concentrations.[5]

The finding of a diminished serum sodium concentration in patients with liver disease and ascites is not uncommon.[18, 19] Thus patients with cirrhosis with varying degrees of fluid retention without neurologic complications have been observed to have mean serum sodium concentrations as follows: those with ascites who have had frequent paracenteses 131.6 mM per liter, those with ascites who have never had a paracentesis 136.7 mM per liter, those without ascites 139.8 mM per liter.[19] Conversely, the diminished serum sodium concentration in individual patients in serial observations had no consistent relationship to the neurological status. Therefore, it appears unlikely that the decreased serum sodium concentration was an important causative factor for the coma, although it may have been contributory in several instances. Although the genesis of the hyponatremia is unclear, the quantity of fluid administered as well as the inability to excrete water may be important. In this regard it is noteworthy that several of these comatose patients had received three to four liters of 5 or 10 per cent glucose solution intravenously daily for several days prior to and during the onset of the comatose state. Furthermore, the administration of increased quantities of water has been shown to produce hyponatremia in some noncomatose patients with cirrhosis of the liver.[20] Although the level of serum electrolytes does not appear to be of prime etiologic importance in the development of hepatic coma, nevertheless rapid acute alterations in serum electrolytes such as may occur following paracentesis may be of contributory significance.

Although severe liver damage is a prerequisite for the development of hepatic coma, no consistent correlation between changes in liver function tests and the development of coma has been evident.[21]

Several other biochemical disturbances have been described in association with hepatic coma. An abnormality in pyruvate metabolism in patients with liver disease has been described by Snell and Butt[6] and by Amatuzio and Nesbitt.[7] Walshe[22] and Dent[23] have reported not only elevations of plasma amino acids but also abnormal concentration patterns in patients with hepatic coma. Among the abnormalities noted, an elevation of plasma glutamine was found in a few patients.

Although many chemical disturbances occur concomitantly with hepatic failure, no one abnormality has been found to be of etiological importance. The alterations in serum electrolytes and pH which occur in association with the comatose state are so inconsistent that they argue against any positive correlation. The disturbance in ammonia metabolism, although not clearly established as being the important causative factor, correlates well enough to warrant further investigation.

SUMMARY

Twenty-two patients with cirrhosis have been observed clinically in various stages of hepatic coma. Serial biochemical measurements of serum pH, carbon dioxide content, and chloride, sodium, and potassium concentrations, and of blood nonprotein nitrogen and ammonia content, and of hematocrit and hemoglobin were made.

Twenty of these patients died in hepatic coma. The presence of a "flapping" tremor was a grave prognostic sign. Characteristic electroencephalographic patterns were found in five of the seven patients in whom tracings were taken.

The mean data indicated no abnormality in acid-base equilibrium of the blood; however, a consistently diminished serum sodium and chloride concentration was noted in the patients with coma. This has been related to the presence of ascites and in some patients to oliguria and abnormally large fluid intakes.

No correlation between individual biochemical abnormalities of sodium, chloride, potassium, or acid-base equilibrium and clinical status was evident. In individual cases, such abnormalities may play a contributory role in the development of hepatic coma.

Elevation of the blood ammonia was noted in patients with cirrhosis of the liver, both with and without impending coma or coma. No precise correlation in serial measurements was noted between blood ammonia level and degree of consciousness.

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SPONTANEOUS ANTIDIURESIS DURING WATER DIURESIS: RENAL FUNCTION AND ELECTROLYTE EXCRETION

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PHYSIOLOGIC diuresis and antidiuresis are generally regarded as being mediated through inhibition or stimulation, respectively, of the neurohypophysis, with consequent diminution or increase in secretion of antidiuretic hormone (ADH). The stimulus for such changes is an alteration in the tonicity of body fluids; dilution associated with the ingestion of large amounts of water leads to diuresis,[1, 2] whereas an increase in osmotic pressure induced by the intake of salt or by dehydration stimulates the secretion of ADH which leads to antidiuresis.[3]

Almost complete inhibition of endogenous ADH with intense diuresis can be evoked and maintained by the rapid ingestion of a large quantity of water followed by the frequent repeated administration of tap water to replace fluid excreted. In a recent study on the action of pitressin in human beings,[4] a subject who was being maintained in a state of marked hydration accompanied by steady diuresis suddenly experienced a period of antidiuresis without apparent cause. Studies were made of renal function and excretion of water and electrolytes. One week later similar studies were made on the same patient to compare the effects of the administration of pitressin with those observed during the spontaneous diuresis.

METHOD OF STUDY

A 34-year-old Puerto Rican woman was admitted to the Mount Sinai Hospital for a Nickerson-Kveim test and for confirmation of a suspected diagnosis of sarcoidosis rather than for any severe symptoms at that time. There was no evidence of cardiac, renal, endocrine, or pulmonary disease.

When the first study was made, she had complained of no symptoms. However, she subsequently stated that she had awakened that day with some pain in the left side of her neck. This was later attributed to an enlarged tender anterior cervical lymph node.

Hydration was carried out orally. The patient received 1,500 ml. of water within a period of thirty minutes. Forty-five minutes later 400 ml. of water was given, and this was repeated in thirty minutes. At this point, the first clearance period was started, and water was subsequently ingested at the rate of 200 ml. every fifteen minutes for the duration of the experiment. In addition the subject received 200 ml. of 0.85 per cent sodium chloride per hour intravenously with the inulin and p-aminohippuric acid infusion which was delivered through a constant infusion pump.

Urine collections were at thirty-minute intervals through an indwelling urethral catheter preceded by suprapubic pressure and an air wash in all instances. Blood samples

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TABLE I. GLOMERULAR FILTRATION AND RENAL PLASMA FLOW

	TIME	CLEARANCES (ML./MIN.)		WATER		SODIUM		CHLORIDE		POTASSIUM
DATE	MINUTES	INULIN	PAH	C.C./MIN.	PER CENT TUBULAR REABSORPTION	MEQ./MIN.	PER CENT TUBULAR REABSORPTION	MEQ./MIN.	PER CENT TUBULAR REABSORPTION	MEQ./MIN.
1/12/53	0-30	112	666	16.3	85.4	277	98.30	227	98.04	65
	30-60	15	84	1.5	90.0	33	98.34	35	97.98	11
	60-90	40	230	2.3	94.3	136	97.75	128	97.00	25
	90-120	231	1498	16.3	92.9	876	97.14	769	96.84	130
1/21/53	0-30	86	520	13.4	84.4	181	98.47	99	98.87	54
	30-60	93	598	14.2	84.8	176	98.65	80	99.16	63
				0.4 Milliunit Pitressin Per Kilogram Intravenously						
	60-75	46	266	2.9	93.7	73	98.85	51	98.92	35
	75-90	93	560	2.7	97.1	113	99.11	115	98.81	51
	90-105	176	1088	10.7	93.9	332	98.63	203	98.86	96
	105-120	89	573	10.7	88.0	193	98.43	99	98.91	64

were obtained from an indwelling venous cannula at the midpoint of each clearance period. Specimens of urine and blood were analyzed for inulin, p-aminohippuric acid, sodium, potassium, and chloride.

The study was conducted in quiet surroundings and assurance was constantly given to prevent emotional factors from modifying the experiment. In spite of these efforts the patient was somewhat apprehensive throughout the entire procedure. However, at approximately three minutes after the start of the second clearance period, the apprehension was markedly increased, characterized by crying, anxiety, pallor, and shivering without rigors. It was at this time that direct questioning revealed the presence of pain in the back of the left shoulder and neck. However, the pain had not changed in intensity and had been constant in duration since onset, that is, before the procedure was started. This episode lasted for about thirty minutes, subsiding gradually. The apprehension tended to fluctuate for the duration of the study but was no more marked in period three than in period four. Studies were discontinued when the urine flows returned to control values.

Nine days later the patient was returned to the laboratory. Hydration was essentially identical with that in the previous study. Clearance studies were carried out at fifteen-minute intervals; blood samples were taken at the midpoint of each period. The experimental data for the control periods have been combined to thirty-minute periods. An intentional attempt to upset the patient did not produce an inhibition of the diuresis. Following control clearance periods, the subject was given 0.4 milliunit of pitressin per kilogram of body weight, by rapid intravenous injection five minutes before the start of the succeeding clearance period. Clearance studies were continued at fifteen-minute intervals for the next seventy-five minutes.

Inulin and p-aminohippuric acid clearances and determinations in plasma and urine were carried out according to the techniques recommended by Goldring and Chasis,[5] except that the inulin was determined by the method of Bojensen.[6] Sodium and potassium determinations were carried out on a Janke flame photometer using lithium as an internal standard. Chlorides were determined by the method of Schales and Schales.[7]

RESULTS

Glomerular Filtration and Renal Plasma Flow.—In the first thirty-minute period of spontaneous antidiuresis the glomerular filtration rate, as measured by inulin clearance, fell sharply from 112 to 15 ml. per minute, but increased to 40 ml. per minute in the next period while antidiuresis persisted. In the fourth thirty-minute period during which the control diuretic rate of flow was restored, glomerular filtration rose well above normal (Table I and Fig. 1).

The renal plasma flow, as measured by PAH clearance, underwent changes corresponding to those of glomerular filtration. The filtration fraction, which was normal (18.7 per cent) during the initial period, remained virtually unchanged throughout the various periods of altered glomerular filtration and plasma blood flow.

The intravenous administration of pitressin induced a primary reduction in glomerular filtration during the first fifteen-minute period of antidiuresis despite maintenance of marked hydration. However, by the next fifteen-minute period the glomerular filtration rate had returned to its normal control value despite the continuation of antidiuresis (Fig. 2). During the third post-pitressin period, glomerular filtration rose to 89 per cent above the control value and returned to the control level in the subsequent period. Renal plasma flow fell 56 per cent from its normal control value in the first fifteen-minute period of antidiuresis, returned to normal in the following fifteen-minute period, and re-

bounded to 82 per cent above normal when the diuresis was resumed. The filtration fraction was slightly, but probably not significantly, increased during antidiuresis.

Water Excretion.—Hydration had induced a diuresis of 16.3 ml. per minute during the first thirty-minute period prior to spontaneous antidiuresis. This indicated a tubular reabsorption of water, amounting to 85.4 per cent, which is approximately the percentage of passive obligatory reabsorption in the

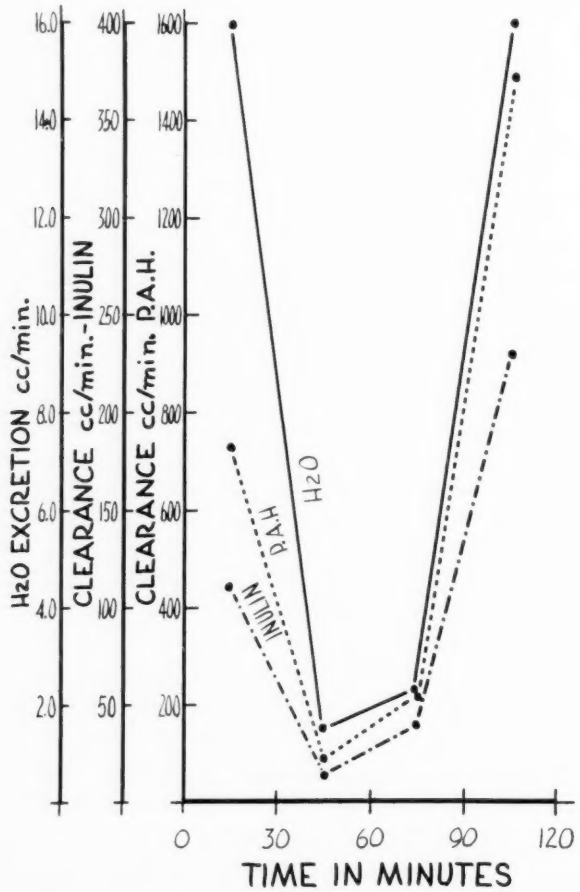

Fig. 1.—Spontaneous antidiuresis. Note that excretion for water and clearances of inulin and PAH are parallel. There is a supernormal rebound phase of inulin and PAH clearance, when diuresis recurs.

absence of any endogeneous ADH.[8] Despite continued hydration, a marked antidiuresis occurred in the second and third periods, the rate of urine flow amounting to 1.5 ml. and 2.3 ml. per minute, respectively, while tubular reabsorption increased to 90 and then to 94.3 per cent. Subsequently the urine flow increased despite a greater percentage and absolute increase in tubular reabsorption, which suggested that ADH was not the only factor concerned (Table I).

Following pitressin antidiuresis occurred promptly, while glomerular filtration and renal plasma flow were markedly reduced in the first fifteen-minute period. This was followed by another period of antidiuresis, although glomerular filtration and renal plasma flow had returned to normal. Subsequent resumption of diuresis was associated briefly with a period of above normal rates of glomerular filtration and renal plasma flow.

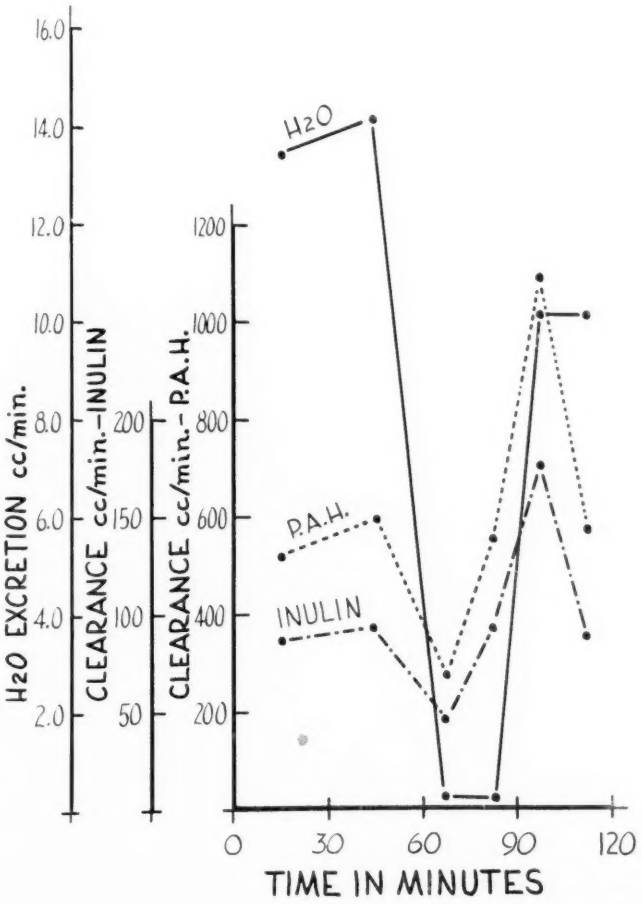

Fig. 2.—Pitressin induced antidiuresis. Note that water excretion remains depressed although clearances for inulin and PAH have returned to normal in the second fifteen-minute post-pitressin period.

Excretion of Electrolytes.—Sodium excretion (rate) fell 88.1 per cent in the first thirty-minute period of spontaneous antidiuresis which corresponded almost exactly to the reduction in glomerular filtration, renal plasma flow, and urine flow. Sodium excretion in the final period, when diuresis recurred, and when glomerular filtration and renal plasma flow were exaggerated, was more than three times as great as during the initial period, when the rate of urine flow was identical at 16.3 ml. per minute (Table I).

When antidiuresis was evoked by intravenously administered pitressin, sodium excretion likewise diminished markedly, corresponding to a similar reduction in glomerular filtration and renal plasma flow. Subsequent excretion of sodium rose to above normal and returned to normal, in accordance with corresponding changes in glomerular filtration and renal plasma flow.

Chloride excretion corresponded essentially with that of sodium during both the spontaneous and pitressin antidiuresis.

Potassium excretion followed a somewhat similar pattern, but the degree of change was quantitatively more striking in association with the spontaneous antidiuresis than with that evoked by pitressin.

DISCUSSION

The occurrence of spontaneous antidiuresis during active hydration and diuresis in this case could not be attributed to the usual physiologic mechanism of increased plasma osmotic pressure with consequent stimulation of ADH. A clue to the possible mechanism of the antidiuresis was sought in a study of renal functions and water and electrolyte excretion before and after the sudden reduction in urinary flow.

The most striking finding was the extreme reduction in glomerular filtration and in renal plasma flow during the first thirty minutes of antidiuresis, suggesting intense renal vasoconstriction. The diminution in filtered water could account for almost all of the reduction in urine flow during this period. The extreme reduction in excretion of sodium and chloride with relatively little increase in concentrations likewise appeared correlated with the fall in glomerular filtration rather than with the endogenous stimulation of ADH. The latter has generally been described as causing an increase in urinary concentration of these electrolytes without significant change in absolute excretion.[9]

The second period of antidiuresis was followed by a thirty-minute period of re-established diuresis during which glomerular filtration and renal plasma flow rebounded to rates greater than twice normal, and electrolyte excretion similarly exceeded their initial normal values. These findings denoted a period of renal vasodilatation following the period of renal vasoconstriction. That urinary flow did not exceed the initial diuresis despite glomerular hyperfiltration was due to higher percentage of tubular reabsorption of water than in the initial period. This may mean that water-loading inhibition of ADH was less complete in this period or that renal vasodilatation was associated with hyperfunction with respect to tubular reabsorption of filtered water.

Although there is no direct evidence to indicate that ADH played any part in the spontaneous antidiuresis, there were certain similarities, as well as differences, between the renal function, water, and electrolyte responses on this occasion and after the administration of a relatively small "physiologic" dose of pitressin. Antidiuresis following the pitressin lasted only two fifteen-minute periods, which was relatively brief compared to the duration usually observed after pitressin was administered to other subjects.[4] During the first fifteen minutes there was a significant fall in glomerular filtration, renal

plasma flow, and excretion of electrolytes, apparently due to renal vasoconstriction.[4] This resembled the period of renal vasoconstriction with the spontaneous antidiuresis. The second fifteen-minute period of antidiuresis was unlike that which occurred spontaneously in that it was independent of changes in glomerular filtration and renal blood flow which were normal, and sodium and chloride excretion values were likewise normal. The high percentage of tubular reabsorption of filtered water also suggested that the antidiuresis during this period was due to the antidiuretic factor present in the injected pitressin without concomitant alteration in blood flow. No such period was observed during the period of spontaneous antidiuresis.

The observation that spontaneous antidiuresis was associated with changes in renal function denoting renal vasoconstriction led to a consideration of the factors which might induce such changes. Vigorous muscular exercise may inhibit water diuresis with concomitant reduction in glomerular filtration rate and renal blood flow.[10, 11] However, the patient with spontaneous antidiuresis was at bed rest during the period of study. Neither phenobarbital[12] nor any other drug[13, 14] capable of evoking antidiuresis was administered.

A possible emotional origin for the antidiuresis was considered because of heightened anxiety at about the time of the onset of the antidiuresis. Emotional factors have been impugned by some as the cause of diuresis[15] and by others as the cause of antidiuresis.[2, 16] A reduction in renal plasma flow without change in filtration rate has been noted in hypertensive patients when life situations of traumatic significance were discussed during psychiatric interviews.[17] However, results with normotensives have been more variable.[18] If emotional factors were solely responsible for the spontaneous antidiuresis in this subject, there was no clear-cut correlation in that the anxiety was present to some degree prior to the antidiuresis and persisted even during the last clearance period with a urine flow of 16.3 per minute.

Painful stimuli in animals have been reported to evoke antidiuresis associated with decreases in both renal plasma flow and filtration rate.[11, 19] The patient in this study did complain of cervical pain through much of the procedure, but her pain was present before and after the spontaneous antidiuresis, and no change in intensity at any time could be detected.

In our case the observations indicated that the production of renal vasoconstriction was the most important factor accounting for the renal effects. The role of the sympathetic nervous system and of endogenous epinephrine was therefore considered. In the dog, Theobald and Verney demonstrated an equal antidiuretic response whether or not the renal nerves are excised or intact.[20] This suggests that a hormonal mechanism is involved. Epinephrine has been reported to cause a marked reduction in renal plasma flow but little or no change in filtration rate since filtration fraction increased.[21] However, Jacobson, Hammarsten, and Heller[22] infused adrenalin by constant injection syringe into twelve young adults and noted a diminution in filtration rate as well as a more marked reduction in renal plasma flow. Sodium chloride and potassium excretion were all reduced, presumably associated with the reduc-

tion in filtration rate. Our observations during spontaneous antidiuresis disclosed somewhat similar findings except that the filtration rate was diminished as much as the renal plasma flow. During spontaneous antidiuresis, there was a slight diminution of tubular reabsorption of sodium and chloride. This was unlike the findings of Smythe, Nickel, and Bradley,[23] who noted augmented tubular reabsorption of electrolytes following epinephrine. However, the interpretation of changing tubular reabsorption in the presence of marked changes of filtration rate becomes difficult. The renal function during the spontaneous antidiuresis observed in our subject most closely resembled the initial phase of pitressin antidiuresis during which there is a diminution in renal plasma flow and glomerular filtration rate associated with the antidiuresis.[4]

The observation of spontaneous antidiuresis in association with renal vasoconstriction and reduction in electrolyte excretion as well as the subsequent rebound renal hyperfunction suggests the importance of considering the possible occurrence of these effects when interpreting observations on diuresis and antidiuresis and on electrolyte excretion in man.

SUMMARY

The occurrence of a spontaneous antidiuresis in a water-loaded normal subject associated with marked changes in renal plasma flow and glomerular filtration is reported.

These changes are compared with those observed after antidiuresis induced by the administration of pitressin.

Possible mechanisms involved in the spontaneous antidiuresis are discussed.

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BIOCHEMICAL AND CLINICAL EFFECTS RESULTING FROM THE ADMINISTRATION OF A CATION-ANION EXCHANGE RESIN IN DECOMPENSATED HEPATIC CIRRHOSIS

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INTRODUCTION

SODIUM retention is a major factor in the pathogenesis of the ascites and edema occurring in patients with cirrhosis of the liver. Antidiuretic[1] and antisaluretic[2] substances produced as a result of faulty hepatic metabolism may exert a direct renal effect leading to retention of salt and fluid. Alterations in serum proteins and portal hypertension may be chiefly concerned with localization rather than formation of ascites and edema.[3]

Recently much attention has been directed to abnormalities of renal function observed in edematous[3-5] and nonedematous[6] patients with hepatic cirrhosis. In 1950, Goodyer and co-workers[3] presented evidence of specific impairment of renal mechanism for the excretion of an intravenously administered 5 per cent sodium chloride solution in patients with ascites and edema. Normal values for mannitol and PAH clearances suggested the retention of dietary sodium due to increased tubular reabsorption by the kidney due to an obscure stimulus. Leslie and associates[5] have noted reductions in filtration rate, effective renal plasma flow, and TmPAH during the phase of rapid accumulation of ascitic fluid. They considered the renal abnormality to be functional and reversible since the inulin and PAH clearances returned to normal following therapy.

Rigid restriction of dietary sodium is often effective in controlling ascites and edema in patients with cirrhosis of the liver.[2, 7, 8] Adequate control of edema by means of sodium depletion may require restriction of dietary sodium to less than 200 mg. per day.[9] Studies by Dock[10] and later by Danowski and co-workers[11] have established the efficiency of cation exchange resins as a means of sodium restriction in edematous states. These resins can interfere with absorption of sodium, potassium, and probably calcium and magnesium from the gastrointestinal tract, and to a much lesser extent remove endogeneous stores of these ions via stool excretion. Studies have been reported suggesting the efficacy of sulfonic[12] and carboxylic cycle cation exchange resins with[13, 14] and without[15] addition of an anion exchanger in patients with cirrhosis of the liver. Toxic manifestations similar to hepatic coma have occurred in patients given ammonium-containing cation exchange resins.[16] Most published reports have been concerned largely with a clinical evaluation of the resins, and contain relatively little data on the long-term effects of resin administration.

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The present report describes the effects of long-term continuous administration of a mixture of cation and anion exchange resins to a group of nine patients with hepatic cirrhosis complicated by ascites and edema. The studies have provided information on the effects of cation resin administration in patients with liver disease in regard to: serum protein, electrolytes, and acid-base balance, liver function tests, histologic appearance of liver tissue, and the clinical course of the disease. Observations are also reported on the relation of edema loss to changes in calculated colloidal osmotic pressure.

METHODS

The subjects of these studies were nine patients with cirrhosis of the liver receiving treatment on the wards of the Louisville General Hospital. All patients had long-standing history of chronic alcoholism with a diet grossly inadequate as regards protein and vitamin intake. All patients had been incapacitated for at least three months with ascites and dependent edema. Frequent paracenteses and the administration of mercurial diuretics had provided only temporary relief of symptoms. Maximal formation of ascites[17] was suggested in most patients by a low serum sodium concentration and twenty-four hour urine sodium excretion while on a regular diet. Pertinent clinical data are summarized in Table I.

The clinical impression of Laennec's cirrhosis was confirmed in all cases by transthoracic liver biopsy by means of a Vim-Silvermann needle. Another biopsy specimen was examined histologically at completion of the study or at autopsy.

A cation-anion exchange resin consisting of 12 per cent alkylene polyamine resin, 29 per cent potassium salt of carboxylic acid resin, and 59 per cent carboxylic acid resin (Carbo-Resin) was administered in three divided doses to a total daily dose of 50 to 70 grams in water. The diet was restricted only by the elimination of the use of salt at the table. The patients were advised to eliminate alcohol, but cooperation in this regard was probably poor. A vitamin B complex supplement was prescribed. No supplemental potassium, calcium, or iron was administered. Initially all patients were bed patients and treated in the hospital. As their condition improved they were encouraged to resume activity and were followed in a special outpatient clinic. Patients were weighed daily in the hospital, and at weekly intervals thereafter. No paracenteses were performed during the treatment period. One patient (W. C.) required mercurial diuretics in addition to the resins.

Serum electrolyte determinations were performed prior to treatment and at weekly intervals thereafter. Blood was collected anaerobically without stasis in oiled syringes and stored under mineral oil. Carbon dioxide and pH determinations were performed on the same blood samples within two hours in all cases. Urine was preserved by addition of toluene and by refrigeration. A Weichselbaum-Varney Universal flame spectrophotometer was used for sodium, potassium, and calcium determinations. Serum sodium was measured at a dilution of 1:500, and potassium and calcium at a dilution of 1:100.

The analytical methods employed were as follows: chloride in serum, method of Sendray modified by Van Slyke and Hiller[18]; carbon dioxide content of serum, Van Slyke and Neill[19]; serum pH, Hastings and Sendray[20]; and by a glass electrode, serum urea, Van Slyke[21]; serum proteins, Wolfson and Cohn[22]; and ammonia in urine, modified Van Slyke and Cullen method.[23]

Colloidal osmotic pressure was calculated by the formula of Wies and Peters,[24] with corrections for water content by formula of Eisenman, MacKenzie, and Peters.[25]

The following determinations were performed as tests of liver function: serum bilirubin,[26] cephalincholesterol flocculation,[27] thymol turbidity,[28] cholesterol-cholesterol ester ratio,[29] prothrombin concentration,[30] and alkaline phosphatase.[31] These tests were performed prior to treatment and at biweekly intervals thereafter.

RESULTS

A. Effects of Treatment on Course of Hepatic Cirrhosis.—Table II presents the data on the effects of a cation-anion exchange resin and a liberal diet on

TABLE I. PRETREATMENT DATA ON PATIENTS

PATIENT	AGE	SEX	RACE	KNOWN DURATION OF DISEASE	DURATION OF ASCITES	NOW JAUNDICED	ESOPHAGEAL VARICES (BY X-RAY)	LIVER SIZE (CM. BELOW COSTAL MARGIN)	SPLEEN SIZE (CM. BELOW COSTAL MARGIN)	SPIDER ANGIOMATA
C. M.	35	F	W	1 yr.	4 mo.	yes	no	7	3	yes
A. H.	44	F	W	5 yr.	24 mo.	no	no	5	2	yes
J. M.	38	M	W	½ yr.	3 mo.	no	yes	4	not palpable	yes
L. R.	52	F	W	2½ yr.	6 mo.	no	no	5	2	no
G. L.	40	F	W	1 yr.	12 mo.	yes	yes	5	not palpable	yes
E. W.	36	M	W	3 yr.	4 mo.	no	yes	7	not palpable	yes
F. H.	66	M	C	5 yr.	36 mo.	yes	yes	8	4	no
W. C.	55	M	W	4 yr.	3 mo.	no	yes	3	not palpable	yes
E. J.	46	M	W	2 yr.	3 mo.	yes	no	6	4	no

the ascites and edema of hepatic cirrhosis. Substantial loss of weight accompanied by the marked decrease or elimination in fluid retention occurred in eight of the nine patients. Edema loss usually occurred rather promptly and was greatest during the first month of treatment. The resin administration was continuous except for brief periods of withdrawal to note the effect on edema. One patient (L. R.) died of postoperative surgical hemorrhage, and one (F. H.) following trauma. One patient (W. C.) responded poorly to treatment and has had a progressive downhill course.

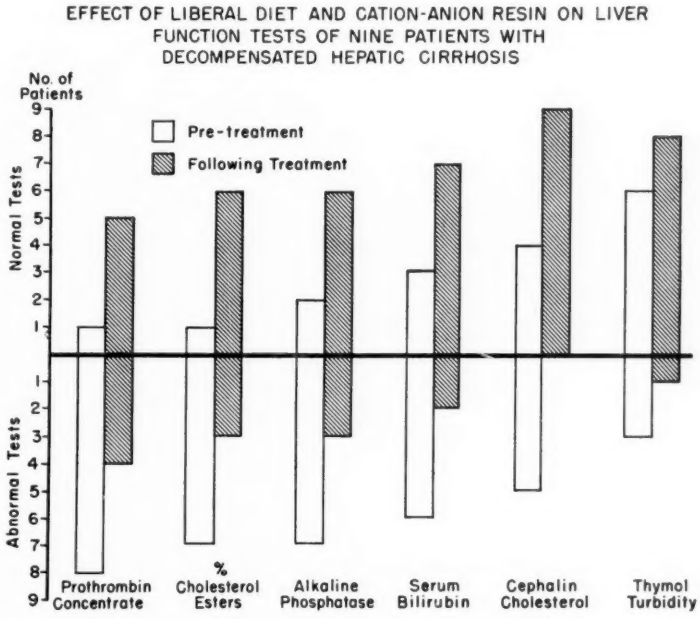

Fig. 1—Effect of liberal diet and cation-anion resin on liver function tests of nine patients with decompensated hepatic cirrhosis.

Histologic studies of liver tissue obtained by transthoracic liver biopsy prior to treatment and by biopsy or autopsy following treatment revealed no significant change in the pathologic pattern of severe Laennec's cirrhosis. Some decrease in intrahepatic fat occurred in the only patient (A. H.) displaying fatty metamorphosis and portal cirrhosis prior to treatment.

B. Effect of Treatment on Liver Function Tests.—Table III and Fig. 1 show changes suggestive of improvement in liver function as revealed by the hepatic tests. In general, such normalization occurred at a much slower rate than loss of edema fluid.

C. Effects of Resin Administration on Serum Constituents and Urinary Sodium Excretion.—Table IV and Fig. 2 reveal that the administration of a cation-anion exchange resin mixture and a liberal diet produced little demonstrable effect on serum sodium, potassium, and chloride levels. (Values greater than 2 S. D. were considered significant.)

TABLE II. DATA ON TREATMENT AND PATIENT RESPONSE

PATIENT	CATION-ANION RESIN ADMINISTRATION (DAYS)	MAXIMUM WEIGHT LOSS (POUNDS)	ASCITES		DEPENDENT EDEMA		REMARKS
			PRE-TREATMENT	FINAL STATE	PRE-TREATMENT	FINAL STATE	
C. M.	372	28	+++	+	+++	0	Excellent response. Free of ascites and edema. Has not required resin for 89 days. Working.
A. H.	372	27	+++	0	+++	0	Excellent response. Free of ascites and edema. Has not required resin for 85 days. Working.
J. M.	340	29	+++	+	+++	+	Good response. Working. Requires resin.
L. R.	120	12	++	0	+++	0	Good immediate response. Died of post operative hemorrhage following pelvic surgery.
G. L.	53	16	+++	0	+++	0	Excellent response. Working. Has not required resin for 180 days.
E. W.	30	30	++++	0	+++	0	Excellent response. Working. Has not required resin for 180 days.
F. H.	178	38	+++	0	+++	0	Excellent response until sudden death from purulent peritonitis following trauma.
W.C.	68	10	++++	++++	+++	+++	Poor response. Requires resin plus 6 c.c. mercurial diuretics per week to remain ambulatory.
E. J.	154	12	+++	0	+++	0	Excellent response. Working. Has not required resin for 82 days.

Serum sodium tended to be low prior to treatment, probably due to serum dilution secondary to the rapid formation of ascites formed out of proportion to available sodium. The diminution of urinary excretion of sodium suggests utilization of a good proportion of dietary sodium for edema formation.

During the initial phase of treatment, a rise rather than the expected fall in urine sodium excretion was noted in four patients. This often occurred during periods of rapid diuresis and weight loss. The rise in urine sodium excretion while the patient was receiving cation exchange resin therapy was most likely the result of the acidifying effects of the resin mixture. During the course of treatment an increase in urine sodium excretion was occasionally noted as the disease process became quiescent and less dietary sodium was utilized for edema formation.

No evidence of an abnormal retention or depletion of the body stores of potassium occurred. The mean changes in serum potassium were not significant. No clinical symptoms or electrocardiographic abnormalities suggestive of potassium deviations from the normal occurred. Serum chloride levels tended to increase in most patients, however the changes were not of great magnitude.

Hypocalcemia did not occur as judged by lowering of serum calcium levels or radiographic changes in bone density. The lowest serum calcium level following prolonged treatment was 5.3 meq. per liter (Patient A. H.).

TABLE III. DATA ON EFFECTS OF TREATMENT ON LIVER FUNCTION TESTS

	SERUM BILIRUBIN (MG. %)		THYMOL TURBIDITY (UNITS)		CEPHALIN-CHOLESTEROL FLOCCULATION FLOC./48 HRS.		PROTHROMBIN ACTIVITY % OF NORMAL		ALKALINE PHOSPHATASE BODANSKY UNITS		% TOTAL CHOLESTEROL IN ESTERIFIED FORM		SERUM UREA NITROGEN (MG. %)	
	PRE-TREAT-MENT	FINAL STATE	PRE-TREAT-MENT	FINAL STATE	PRE-TREAT-MENT	FINAL STATE	PRE-TREAT-MENT	FINAL STATE	PRE-TREAT-MENT	FINAL STATE	PRE-TREAT-MENT	FINAL STATE	PRE-TREAT-MENT	FINAL STATE
C. M.	5.3	0.8	1.7	1.00	+ +	neg.	60	90	9.7	9.4	---	---	4.0	7.88
A. H.	0.60	0.42	6.17	4.98	+ + + +	+ +	50	58	5.9	7.3	60.6	83.7	5.0	7.05
J. M.	4.17	1.6	1.74	1.74	+ + + +	+ +	38	20	15.5	7.0	60.1	62.1	7.6	12.34
L. R.	0.60	0.64	1.3	0.37	+ +	+	12	85	10.3	5.2	61.4	83.4	13.6	10.56
G. L.	1.9	0.70	4.65	2.5	+ + +	+	14	79	7.4	5.76	71.0	86.2	13.2	18.12
E. W.	2.12	0.50	1.09	1.00	+ + +	+	60	85	13.9	5.2	53.2	79.8	12.2	14.25
F. H.	2.45	0.80	6.0	3.48	+ +	+ +	14	7	17.3	15.1	58.5	61.5	13.0	13.41
W. C.	0.70	1.00	1.09	2.09	+	+ +	38	65	14.6	11.0	60.1	45.0	12.2	16.01
E. J.	2.34	0.60	0.87	0.87	+ + +	+	80	100	9.9	7.8	40.8	83.9	10.6	10.08

TABLE IV. EFFECT OF ADMINISTRATION OF CATION-ANION RESIN MIXTURE ON SERUM ELECTROLYTE AND ACID-BASE BALANCE

	SERUM SODIUM (MEQ/L.)			SERUM POTASSIUM (MEQ/L.)			SERUM CHLORIDES (MEQ/L.)			SERUM CO_2 CONTENT (MEQ/L.)			SERUM pH			URINE SODIUM EXCRETION MEQ./24 HOURS	
	CONTROL	R_x	S.D.	CONTROL	R_x	S.D.	CONTROL	R_x	S.D.	CONTROL	R_x	S.D.	CONTROL	R_x	S.D.	CONTROL	8TH DAY RESIN THERAPY
C. M.	137.5	137.14	4.38	4.10	5.29	0.82	95.6	100.35	2.49	26.15	23.21	2.15	7.37	7.368	0.008	158.83	97.015
A. H.	134.0	138.78	5.07	4.46	4.76	0.78	102.0	102.24	2.42	25.05	24.10	1.53	7.395	7.390	0.040	91.50	30.09
J. M.	133.8	134.86	4.95	5.50	5.18	0.71	96.3	102.29	3.01	22.00	22.02	1.64	7.35	7.369	0.025	3.7	42.6
L. R.	138.7	133.2	3.48	4.30	4.87	0.49	101.0	99.36	3.76	25.15	22.71	2.67	7.42	7.366	0.024	14.2	22.7
G. L.	134.8	132.46	2.69	3.90	4.56	0.53	99.0	95.39	4.04	25.75	26.95	1.30	7.41	7.393	0.002	14.7	19.9
E. W.	132.5	131.9	3.84	5.20	5.12	0.50	98.8	99.90	2.84	22.95	23.70	1.21	7.395	7.390	0.055	7.2	51.2
F. H.	135.0	135.09	6.76	4.49	4.37	0.40	102.5	103.34	2.08	27.70	23.00	2.56	7.395	7.386	0.017	166.4	41.2
W. C.	136.6	138.83	5.47	4.80	5.13	1.50	94.8	99.87	9.19	29.00	25.29	2.52	7.415	7.404	0.002	0.7	0.7
E. J.	131.5	134.88	4.57	6.10	5.74	1.85	87.7	91.15	3.99	32.10	30.19	2.04	7.40	7.385	0.32	84.2	84.9

R_x columns represent the mean of the weekly determinations during the resin mixture administration.

The resin administration produced a lowering of carbon dioxide content which was often accompanied by a fall in serum pH. These changes as a group represent a metabolic acidosis which was unaccompanied by any clinical symptoms. Urinary ammonia formation increased in each instance following resin administration (mean increase of 132.69 me.q. of ammonia nitrogen per twenty-four hours).

D. *Effect of Treatment on Serum Proteins and Colloidal Osmotic Pressure.*—Total serum proteins increased in all patients, and this was accompanied by a rise in the albumin fraction in seven of the nine patients as shown in Table V. Calculated colloidal osmotic pressure showed a mean increase of 31 per cent (range 8.96 per cent to 81.13 per cent) during the treatment period. Body weight changes became a poor index of the state of edema and ascites, since patients tend to replace edema fluid with flesh as treatment progresses.

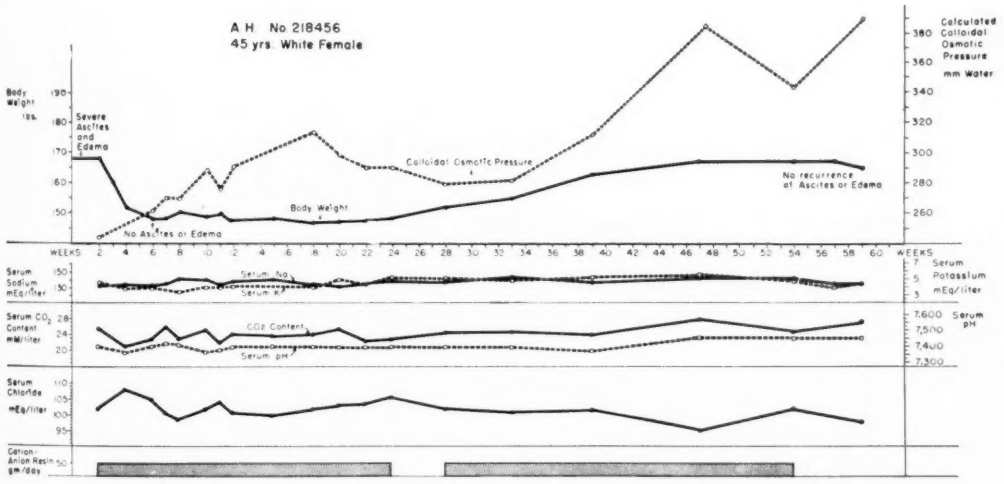

Fig 2.—Long-term effect of administration of cation-anion resin mixture. Note relatively small changes in serum sodium, potassium, and choride. Serum pH and carbon dioxide content generally changed in a parallel manner.

It should be noted that during short-term periods of observations marked fluctuations occurred in body weight accompanied by evident change in amount of ascites and edema present without any appreciable change in serum oncotic pressure. As shown in Fig. 3, patient J. M.'s weight increased 10 pounds accompanied by a decrease of 3.49 mm. of water in calculated oncotic pressure. When the resin was again administered his weight decreased 20 pounds, while oncotic pressure decreased 9.56 mm. of water.

DISCUSSION

The resin mixture administered in this study contained acidifying (hydrogen cycle), nonacidifying (potassium cycle) carboxylic resin with an anion exchanger. Martz and collaborators,[14] using a similar mixture, have demonstrated

an increased efficiency in regard to fecal excretion of sodium following the addition of the anion exchanger. Stool sodium was increased by approximately 30 per cent. Greenman and co-workers[32] have confirmed this increase in efficiency but point out this amounts to only 0.2 milliequivalents per gram of administered carboxylic acid resin and doubt its clinical significance.

Marked alterations in acid-base balance did not occur in this study. Lowering of carbon dioxide content and serum pH was usually of small magnitude, and never accompanied by clinical symptoms. There are at least two possible explanations for the lessened incidence of marked acidosis: (a) fecal excretion of anion, principally phosphate, increases with the cation-anion mixture, so that less anion load is excreted by the kidney. Urinary ammonia increased in all patients following resin administration, so that deviation of concentration of

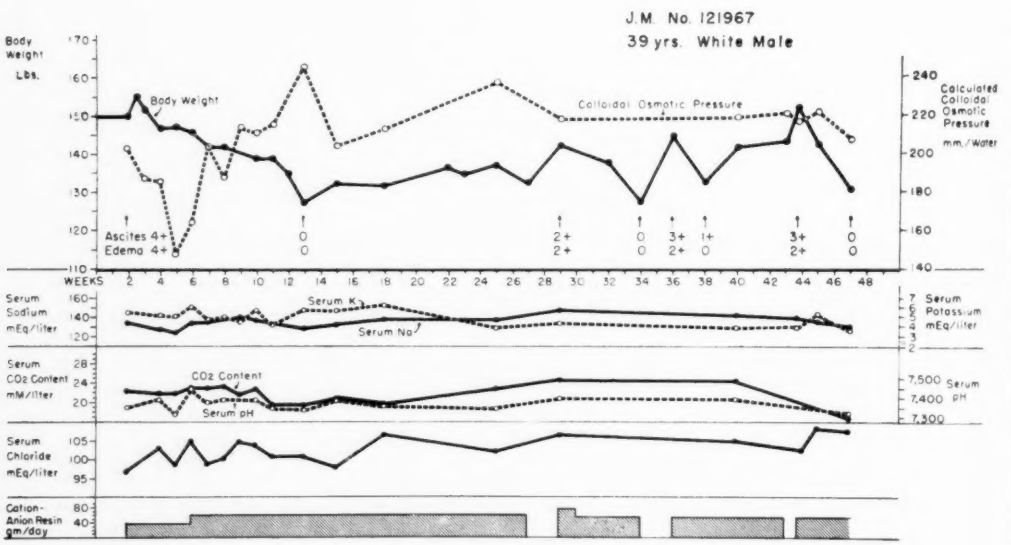

Fig. 3—Long-term effects of cation-anion resin administration. Note poor correlation between acute changes in colloidal osmotic pressure and body weight.

anion in blood did not occur. (b) Less acidifying type resin was ingested. The lessened incidence of a marked metabolic acidosis is not necessarily an advantage in patients with good renal function, since administration of drugs such as ammonium chloride which result in acidosis and increase in serum chloride are useful in treating sodium and water retention.[11]

The data would seem to indicate the importance of electrolyte equilibrium in the genesis of the ascites and edema associated with hepatic cirrhosis. Improvement on resin therapy suggests the importance of sodium retention. Little relationship was noted between changes in edema or ascites and calculated colloidal osmotic pressure during short-term periods of study.[33] In individual patients weight increased as much as 10 pounds or decreased as much as 20

TABLE V. EFFECT OF TREATMENT ON SERUM PROTEINS, CALCULATED COLLOIDAL OSMOTIC PRESSURE AND BODY WEIGHT

	SERUM ALBUMIN (GM. %)			SERUM TOTAL PROTEIN (GM. %)			COLLOIDAL OSMOTIC PRESSURE		BODY WEIGHT	
PATIENT	CONTROL	R_x	S.D.	CONTROL	R_x	S.D.	PRE-TREATMENT (MM. WATER)	POST TREATMENT (% CHANGE)	PRE-TREATMENT (LB.)	POST TREATMENT (% CHANGE)
C. M.	3.82	4.31	0.445	6.86	7.73	0.604	269.7	+41.08	139	+ 8.63
A. H.	2.86	3.50	0.36	7.37	7.99	0.036	244.3	+27.71	168	− 1.48
J. M.	2.22	2.07	0.47	6.70	6.88	0.51	203.0	+ 8.96	155	− 8.22
L. R.	3.35	3.86	0.213	5.99	6.93	0.39	230.3	+20.40	143	+ 0.69
G. L.	1.30	2.72	0.62	7.30	7.93	0.230	181.8	+81.13	120	− 8.33
E. W.	3.06	4.04	0.24	5.95	8.46	0.52	218.0	+35.32	158	−18.35
F. H.	1.45	1.85	0.21	5.52	6.53	0.550	141.6	+24.64	212	−13.20
W. C.	2.75	2.71	0.87	5.99	6.75	0.58	206.6	+19.36	170	−11.18
E. J.	3.05	3.77	0.31	6.74	7.36	0.47	230.3	+20.40	120	+ 0.69
Mean	2.65	3.20		6.49	7.40		225.06	+31.00	153.88	− 5.63

pounds without significant changes in serum protein concentrations or estimated oncotic pressure. It is of interest that all patients demonstrated increases in colloidal osmotic pressure over pretreatment levels during the entire period of observation. It seems unlikely that anatomic changes occurred in the liver to account for the observed rapid diuresis. No histologic improvement was noted in serial liver biopsy specimens.

The reasons for the marked improvement in liver function as evaluated by clinical symptoms and hepatic tests are not clear. The elimination of ascites and edema may have resulted in an improved nutritional state by virtue of improvements in appetite, economic status, or possibly by increased efficiency of absorption across the less edematous mucosa of the gastrointestinal tract. It should be noted that the widely used Patek regime[34] for the treatment of Laennec's cirrhosis utilized sodium restriction in patients with ascites and edema. Our results in the small series of patients suggest that an abnormally high protein diet is not necessary provided sodium retention is eliminated.

The changes in serum sodium, potassium, and chloride were not striking. The initial low serum sodium concentrations noted prior to treatment probably represent dilution secondary to the marked fluid retention. The post-treatment serum sodium values may actually represent a lowering of body sodium since the factor of dilution has been largely eliminated. The production of a true hyponatremic state seems unlikely during ingestion of the resin and a liberal diet due to recognized inability of resins to affect endogenous body stores of sodium. A smaller proportion of exchange capacity is utilized in fecal excretion of potassium in an unrestricted diet, so potassium depletion seems unlikely. The absence of hyperchloremia is possibly related to the decreased tendency toward acidosis.[32]

No recognized alterations in serum calcium or evidence of vitamin deficiency occurred during the period of observation. Serum magnesium determinations were attempted, but difficulty with the method precluded their evaluation.

Studies by Martin and colleagues[35] suggest the possibility of hypomagnesemia following the administration of a cation exchange resin concomitant with a diuresis. Magnesium deficits in experimental animals produce profound physiologic and pathologic changes which presumably could be important in man. Recently attention has been called to low serum magnesium levels in portal cirrhosis.[36]

The anion-cation exchange resin mixture demonstrated its efficiency in the management of fluid retention in certain cases of hepatic cirrhosis. It was well tolerated and rarely accompanied by gastrointestinal disturbances. The data offers no information as to any increase in efficiency following the addition of the anion exchanger to the resin mixture, however a low incidence of severe acidosis was noted.

SUMMARY

The administration of an anion-cation exchange resin mixture is described in nine patients incapacitated by ascites and edema associated with hepatic cirrhosis. Improvement as defined by loss of ascites and edema, normalization of liver function tests and an improved clinical state was observed in the majority of patients.

Long-term administration of the resin mixture with a liberal diet was accompanied by no striking changes in the serum concentrations of sodium, potassium, chloride, and calcium.

Serum pH and carbon dioxide content tended to decrease in a somewhat parallel manner during treatment. The metabolic acidosis was not as severe as has been described elsewhere.

No significant relationship was noted between short-term changes in fluid retention and colloidal osmotic pressure. The relationship of sodium retention to ascites and edema in Laennec's cirrhosis is reaffirmed.

Serum proteins and colloidal osmotic pressure increases noted during the long-term study probably represent an improved nutritional state of the patients.

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THE QUANTITATIVE AND QUALITATIVE RESPONSES OF BLOOD LYMPHOCYTES TO STRESS STIMULI

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WHEN adrenalectomized animals are subjected to stressors, a lymphocytosis occurs which is greater than that which characteristically follows adrenalectomy alone.[1] The time of onset and the rate of increase of the number of circulating lymphocytes of such stressed adrenalectomized animals form a curve which is the inverse image of the lymphopenic response of intact animals known to be mediated by adrenocortical hormones[2] and, therefore, has been termed the lymphocytotic response.

In addition to the changes in number of lymphocytes, microscopic examination of the blood films taken from adrenalectomized and stressed adrenalectomized mice revealed that many of the blood lymphocytes of these animals had undergone distinctive morphologic alterations as compared to the lymphocytes of intact nonstressed mice.

Experiments were designed by which the morphologic and numerical lymphocyte responses produced by stress could be compared.

MATERIALS AND METHODS

Twenty male CBA mice, twelve to sixteen weeks of age, whose body weights ranged between 26 and 28 Gm. were used. Epinephrine HCl (aqueous 1:1,000) was employed as a nonspecific stress stimulus and diluted so that 0.1 ml. contained 0.2 γ for intraperitoneal injection. Adrenalectomy was performed under ether anesthesia by the usual dorsal approach one hour prior to the experiment.

Tail blood samples for total leukocyte counts and differentials were taken prior to epinephrine injection (one hour after adrenalectomy) and at ten, thirty, sixty, one hundred and twenty, and two hundred and forty minutes after injection. Blood films were stained with May-Grünwald Giemsa, and differential leukocyte counts performed on a minimum of 200 cells with at least two observers contributing counts to each experimental group of animals. Lymphocytes were classified as normal lymphocytes and hyaline (or "stress" lymphocytes[3]).

The experimental procedures were performed on the following groups of animals: (1) Intact mice given 0.2 γ epinephrine intraperitoneally and (2) mice given 0.2 γ epinephrine intraperitoneally one hour after adrenalectomy. Control values consisted of total leukocyte counts and absolute

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TABLE I. ABSOLUTE LEUKOCYTE VALUES IN THOUSANDS AND STANDARD ERRORS OF NORMAL, INTACT MICE, MICE 60 MINUTES AFTER ADRENALECTOMY AND INTACT AND ADRENALECTOMIZED MICE GIVEN 0.2 γ EPINEPHRINE I.P. 60 MINUTES AFTER ADRENALECTOMY

GROUPS	NO. OF MICE	TIME	W.B.C.		NORMAL LYMPHOCYTES		STRESS LYMPHOCYTES		TOTAL LYMPHOCYTES		POLYMORPHONUCLEARS		EOSINOPHILS	
			MEAN	S.E.	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.	MEAN	S.E.	MEAN	S
Control														
Normal intact	40	—	11.3 ± 0.6		6.6 ± 0.4		1.3 ± 0.1		7.9 ± 0.6		3.2 ± 0.3		0.3 ± 0.1	
Adrenalectomized	25	60 min.	26.4 ± 1.7		9.1 ± 0.6		2.8 ± 0.3		11.9 ± 0.6		13.7 ± 1.3		0.8 ± 0.1	
Experimental														
Intact mice 0.2 epinephrine i.p.	10	10 min.	14.8 ± 1.3		6.5 ± 0.6		2.2 ± 0.2		8.7 ± 0.5		5.8 ± 1.5		3.8 ± 1.1	
	10	30 min.	15.5 ± 3.1		2.6 ± 0.5		1.7 ± 0.3		4.3 ± 0.6		11.0 ± 2.6		2.4 ± 0.7	
	10	60 min.	19.3 ± 1.7		4.7 ± 0.7		1.5 ± 0.3		6.2 ± 0.7		12.8 ± 1.8		2.7 ± 0.7	
	10	120 min.	21.1 ± 3.5		4.4 ± 1.1		1.3 ± 0.2		5.7 ± 1.2		15.3 ± 2.3		1.8 ± 0.6	
	10	240 min.	40.7 ± 3.7		5.2 ± 0.9		4.2 ± 0.8		9.4 ± 1.7		31.0 ± 2.8		1.5 ± 0.8	
Adrenalectomized mice 0.2 epinephrine i.p.	10	10 min.	22.5 ± 3.4		8.1 ± 0.2		5.1 ± 2.2		13.2 ± 2.3		8.0 ± 1.5		1.7 ± 0.7	
	10	30 min.	20.1 ± 0.7		5.3 ± 0.4		3.1 ± 0.6		8.4 ± 0.8		11.3 ± 1.5		3.6 ± 1.0	
	10	60 min.	21.0 ± 1.7		4.4 ± 1.5		4.3 ± 0.8		8.7 ± 1.2		11.2 ± 1.7		2.0 ± 1.2	
	10	120 min.	27.2 ± 5.2		7.7 ± 2.2		5.4 ± 2.0		13.1 ± 2.8		14.0 ± 1.3		5.7 ± 0.3	
	10	240 min.	38.3 ± 5.1		12.2 ± 2.6		8.2 ± 2.2		20.4 ± 4.3		17.7 ± 2.3		3.3 ± 1.9	

numbers of the various leukocytes determined on a group of 40 intact nonstressed mice and a group of 25 nonstressed adrenalectomized mice one hour after operation.

RESULTS

Total leukocyte counts and the absolute numbers of the different leukocytes of untreated-intact and untreated-adrenalectomized mice one hour after operation are presented in Table I. A significant lymphocytosis of both normal and stress lymphocytes occurred one hour following adrenalectomy.

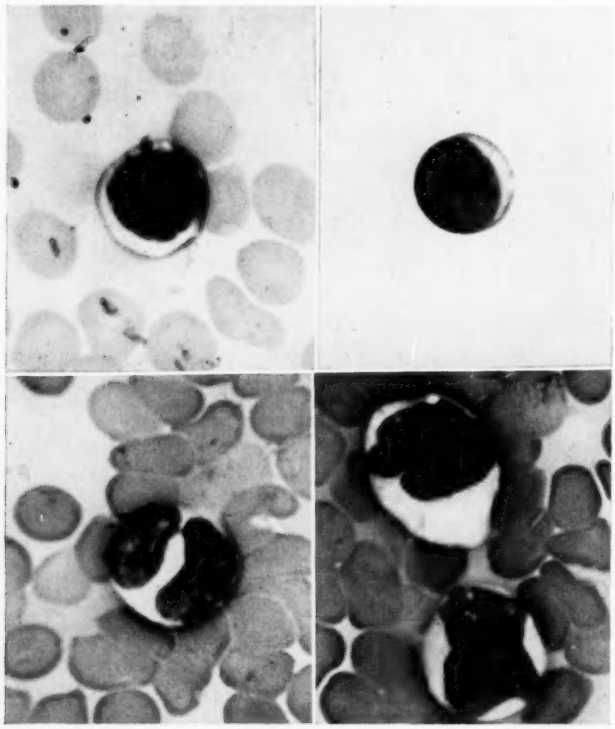

Plate I.—*A* and *B*, Normal mouse lymphocytes; *C*, stress lymphocytes with bilobed nucleus; *D*, stress lymphocytes.

A morphologic comparison of normal mouse lymphocytes and stress lymphocytes is presented in Plate I. The most constant morphologic alterations of stress lymphocytes as compared to normal cells were increased cytoplasmic-nuclear ratios and nuclear and cytoplasmic distortion. The large amount of cytoplasm was poorly basophilic and presented a hyaline appearance. Nuclei were round, polymorphous, or bilobed. The chromatin was found to be aggregated in small discrete clumps.

The curve of the lymphocytic response to stress stimuli may be divided into three phases (Fig. 1). Phase I is characterized by a lymphocytosis, and

Phase II by a lymphopenia (first demonstrated by Michael[4]). Reconstitution of the number of circulating lymphocytes to normal or greater than normal levels forms a third portion of the curve and is termed here Phase III. These categorizations provide a useful means for describing lymphocyte response curves.

A comparison of the lymphocyte responses of intact and adrenalectomized mice given 0.2 γ epinephrine is presented in terms of total lymphocytes, normal lymphocytes, and stress lymphocytes (Fig. 2). The bars represent the total numbers of lymphocytes, the stippled portion the number of stress lymphocytes, and the clear portion the number of normal lymphocytes. Standard errors are presented in Table I.

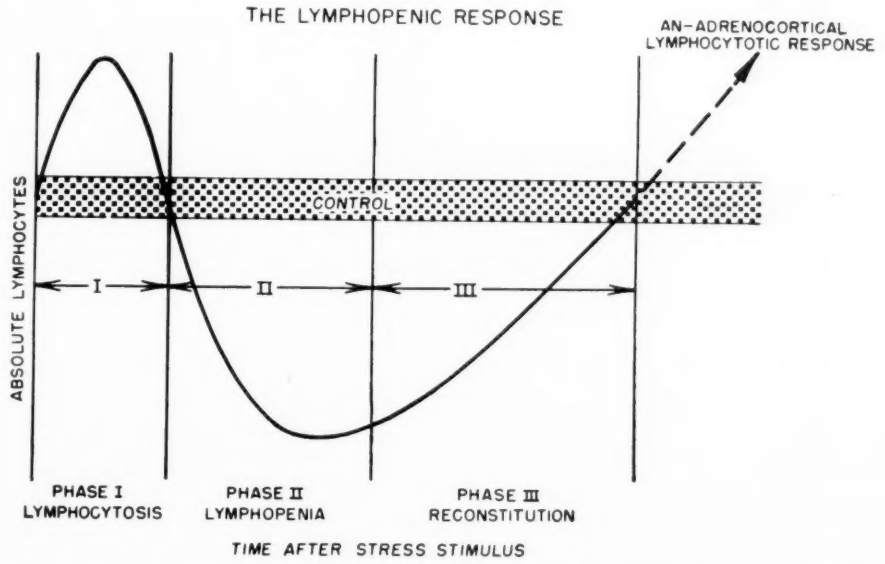

Fig. 1.—A diagrammatic representation of the lymphopenic response of intact animals following stress.

In the intact mice the lymphocytosis characteristic of Phase I did not occur. The lymphopenia of Phase II occurred at thirty minutes, and reconstitution (Phase III) by the end of the experimental period (two hundred and forty minutes). With the exception of a moderate rise in numbers of stress lymphocytes in Phase III there was no significant fluctuation in numbers of these cells at any other time. Both the lymphopenia and the reconstitution were entirely due to changes in numbers of normal lymphocytes.

In the adrenalectomized mice the curve of the total lymphocyte response was nearly identical in configuration to that of the intact mice. In terms of total lymphocytes, the lymphocytosis of Phase I was not significant; there was a relative lymphopenia in Phase II when compared to the adrenalectomized controls, although not a true lymphopenia if compared to the lymphocyte values for normal intact mice. Phase III was represented by a reconstitution

and a lymphocytotic overshoot. During Phase II numbers of normal lymphocytes decreased significantly but returned to control values in Phase III. The most marked change was that of a gradual, significant increase in the numbers of stress lymphocytes throughout all phases.

Similar observations were noted when adrenalectomized mice were subjected to other types of stress stimuli, e.g., fasting, exposure to cold, anaphylaxis, histamine administration, and immunization to horse serum. Since the numerical and morphologic alterations occurring after these stress stimuli were similar to those described here, it is not deemed necessary to describe the results of each of these experiments.

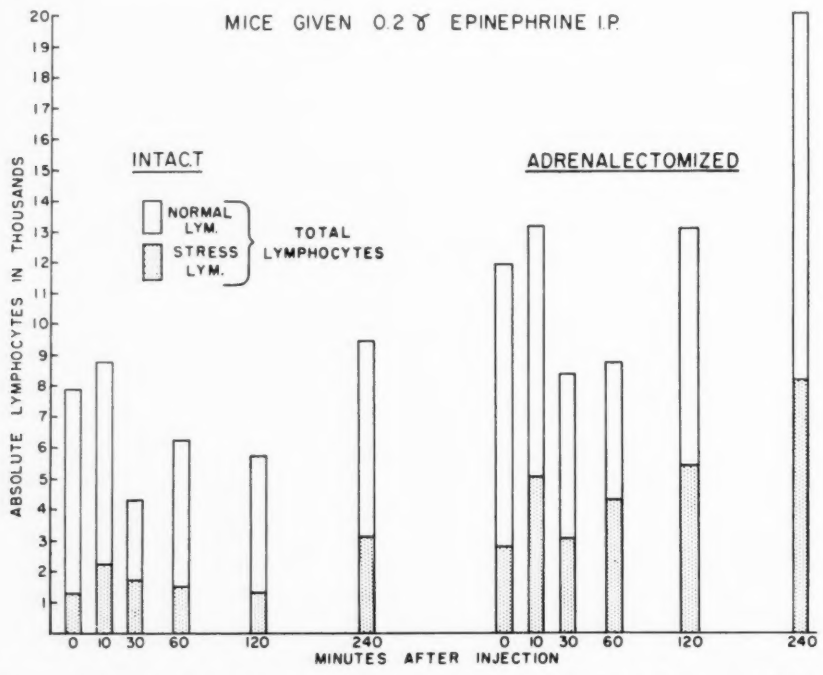

Fig. 2.—Comparison of the absolute numbers of lymphocytes of intact and adrenalectomized CBA mice following injection of 0.2 γ epinephrine intraperitoneally.

DISCUSSION

It is apparent from the data presented here that two morphologic types of lymphocytes exist in the normal intact mouse. The predominant form is the usual small, round lymphocyte exhibiting heavy nuclear chromatin and rimmed by a small amount of cytoplasm. Another type normally present in small numbers is a larger cell characterized principally by an increased amount of poorly basophilic cytoplasm. These cells have been termed stress lymphocytes since they are characteristically increased in number following stressors. It is evident from the data presented here that the total numerical lymphocyte response masks the differences of numerical response that exist between these two different types of lymphocytes, whether or not the response is adrenocortically mediated or nonadrenocortically mediated.

In these experiments, epinephrine was used as a nonspecific stressor, since it has been shown that epinephrine administration results in enhanced secretion of adrenocortical steroids.[5] Since the lymphopenia observed in Phase II in stressed intact mice did not occur in stressed adrenalectomized mice, this response is adrenocortically mediated and confirms the previous report of Gellhorn and Frank.[6] It should be emphasized as demonstrated here that the lymphopenia was due primarily to a decrease in numbers of normal lymphocytes, whereas the numbers of stress lymphocytes remained relatively constant. This finding suggests that normal lymphocytes are more susceptible to the well-known lymphocytolytic action of adrenocortical hormones than stress lymphocytes. This possibility is strengthened by the finding that administration of ACTH or cortisone to human subjects results in a lymphopenia of normal but not stress lymphocytes.[7]

Since circulating lymphocytes do not increase beyond a certain level following adrenalectomy,[1-8] a true lymphocytotic response is defined as an increase in numbers of lymphocytes to values significantly higher than those observed following adrenalectomy alone.[1]

The relative lymphopenia of Phase II (as compared to adrenalectomized control levels) in the stressed adrenalectomized mice was due to a decrease in the number of normal lymphocytes. Thus, although stress is not accompanied by a lymphopenia in adrenalectomized animals when the number of cells is compared to that of intact controls, it may be seen that a small decrease of lymphocytes does occur in Phase II even in the absence of the adrenal cortex. The outstanding characteristic of the lymphocyte response following epinephrine given to adrenalectomized mice was that of a marked lymphocytotic overshoot in Phase III, principally due to a significant increase in numbers of stress lymphocytes.

It is clearly demonstrated here that although adrenocortical secretions tend to decrease the number of circulating lymphocytes, absence of these hormones does not necessarily imply hyperactivity of those forces tending to increase the number of circulating lymphocytes. Kumagai and Dougherty[9] have found that the degree of the lymphocytotic response following a stress stimulus (histamine) in adrenalectomized mice was inversely proportional to the amount of cortisone given as prestress maintenance treatment.

In the intact animal stress stimuli effect two responses of lymphatic tissue: (1) the well-known lymphopenic response mediated by adrenocortical secretions, and (2) a nonadrenocortically mediated response (lymphocytotic) which tends to increase the number of lymphocytes. The results reported here contribute the additional evidence that following stress a lymphopenia is a result of destruction of normal lymphocytes and that the subsequent lymphocytosis is primarily the result of an increase in the numbers of stress lymphocytes. Therefore, it is felt that a normal distribution of blood lymphocytes reflects a balance between the adrenocortically mediated and nonadrenocortically mediated effects of stress on lymphatic tissue.

Histologic studies of lymph nodes of normal intact mice, mice which have been chronically treated with ACTH or cortisone[10] and stressed adrenalecto-

mized mice[11] indicate that the lymphocytes of these lymphatic organs undergo similar alterations as do blood lymphocytes. The lymphatic tissue from hormone-treated mice reveals inhibition of mitosis and a monotony of small pyenotic lymphocytes.[10] The lymphatic tissue from stressed adrenalectomized mice is characterized by increased mitosis[1, 11] as compared to nonstressed adrenalectomized mice. It appears then that the lymphocytosis found in stressed adrenalectomized mice may be attributed partially to the concomittant increase in mitotic rate in the lymphatic tissue. The increase in number of stress lymphocytes could be due to a direct stress mediated effect on pre-existing normal lymphocytes or to a stimulation of development of this altered form from the reticuloendothelial lymphocyte precursors. Although this problem is still under investigation, the rapid increase in the stress lymphocytes following stressors indicates that at least some pre-existing normal lymphocytes were altered.

SUMMARY

Morphologic and numerical lymphocyte responses of stressed intact and adrenalectomized mice were studied.

Two morphologic types of lymphocytes exist in the normal intact mouse: (1) normal lymphocytes, and (2) stress lymphocytes which are large cells with increased amounts of poorly basophilic cytoplasm.

The lymphopenia induced by adrenocortical mediation of stress in intact mice was due to a decrease in numbers of normal lymphocytes, whereas the stress lymphocytes did not decrease or underwent an actual increase. It is thus apparent that stress lymphocytes are more resistant to the lytic effects of adrenocortical hormones than normal lymphocytes.

The lymphocytosis induced by stress in adrenolectomized mice was due primarily to an increase in numbers of stress lymphocytes.

The production of stress lymphocytes is due to nonadrenocortically mediated effects of stressors which bring factors into play which increase the number of lymphocytes and induce specific cytologic changes.

The authors wish to thank Miss Katherine Seymour for her excellent technical assistance.

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THE ASSESSMENT OF STRESS IN HUMAN SUBJECTS BY MEANS OF QUANTITATIVE AND QUALITATIVE CHANGES OF BLOOD LYMPHOCYTES

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PREVIOUS investigations in this laboratory on the circulating lymphocytes of mice suggest that, numerically and morphologically, a normal lymphocyte picture represents a balance between an adrenocortical hormonally mediated effect which tends to decrease blood lymphocytes and causes the morphologic alterations of pyknosis, cytoplasmic budding, and karyorrhexis and a nonadrenocortically mediated effect which increases blood lymphocyte levels and produces the morphologic alterations of hyalinization of cytoplasm, cellular distortion, and increased cytoplasmic-nuclear ratio.[1-5]

Morphologically, these "stress" lymphocytes which are present in increased numbers in stressed, adrenalectomized mice[4] are identical to the Type II lymphocytes described by Downey and associates in human beings.[6] These lymphocytes do not appear solely in infectious mononucleosis as originally described, but have been found in the blood of normal subjects and in patients with a wide variety of disease entities.[7-15]

Observations on the lymphocytes of untreated-normal subjects, normal subjects given ACTH, and normal subjects given epinephrine were made and correlated with blood levels of 17-hydroxycorticosteroids. In addition, the lymphocytic changes accompanying advanced cirrhosis, infectious mononucleosis, tuberculosis, and other disease entities were studied.

MATERIALS AND METHODS

Total leukocyte and differential counts were determined on capillary blood. Cover-slip preparations were stained with May-Grünwald Giemsa. At least two observers contributed differential counts from each cover slip. Leukocytes were classified as follows: segmented neutrophils, nonsegmented neutrophils, normal lymphocytes, Type I lymphocytes (Downey), Type II lymphocytes (Downey), monocytes, eosinophils, and basophils.

Blood 17-hydroxycorticosteroid levels were determined by Dr. D. H. Nelson and Dr. A. A. Sandberg using the technique of Nelson and Samuels.[16]

Values for untreated normal subjects were obtained from the total leukocyte and differential counts of 54 university students.

Experimental groups were: (1) ten normal subjects given 15 mg. of ACTH intravenously. Blood samples were taken at fifteen, sixty, one hundred

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and twenty, and two hundred and forty minutes after injection. (2) Two normal subjects given 3 mg. of epinephrine in an infusion of 500 ml. of 5 per cent glucose during a four-hour period. Blood samples were taken at zero, one, two, three, four, six, and eight hours.

Clinical cases included advanced cirrhosis, infectious mononucleosis, and patients with tuberculosis. Blood films and the results of leukocyte counts from 30 patients with tuberculosis were sent to our laboratory for evaluation.* These patients were placed in three categories: (1) active and untreated, (2) active and under treatment, and (3) well. The blood films sent were designated simply as groups 1, 2, and 3, and clinical evaluation was not revealed until the hematologic study was completed. Evaluation of the lymphocyte response of patients with tuberculosis was based upon the ratio of the absolute number of Type II lymphocytes to the absolute number of normal lymphocytes. This ratio is hereafter designated as the lymphocytic index. The lymphocytic indices were then compared with the lymphocytic index of a group of untreated control subjects with the assumption that an increased index (more stress lymphocytes, fewer normal lymphocytes, or a combination of both) reflected greater stress.

RESULTS

A comparison of the morphologic characteristics of normal lymphocytes, Type II lymphocytes, "stress" lymphocytes (human), "stress" lymphocytes (mice) and monocytes is seen in Plate I. For all practical purposes, the morphologic characteristics of the Type II lymphocyte, the human stress lymphocyte, and the mouse stress lymphocyte are similar. The cytoplasmic-nuclear ratio is increased, and there is often cellular distortion. The chromatin is dense, coarse, and aggregated into discrete clumps. The cytoplasm is poorly basophilic (hyaline) and may be vacuolated and contain azurophilic granules. There is a loss of the perinuclear clear zone common to normal lymphocytes. These cells are easily distinguished from monocytes which have finely reticulated chromatin, folding of the nucleus, and a grayish cytoplasm containing fine azurophilic particles.

A comparison of the absolute values for the various leukocytes of normal males and females studied here with those from normal males studied by Havens[11] is seen in Table I. The differences between males and females of

TABLE I. MEAN VALUES OF LEUKOCYTES IN NORMAL SUBJECTS

		PMN			LYMPHOCYTES					
	NO.	TOTAL WBC	SEG-MENTED	NON SEG-MENTED	NORMAL	I	II	TOTAL	MONO-CYTES	EOSIN-OPHILS
Males*	27	8860	5140	140	1790	160	750	2700	480	170
Females*	27	8420	4720	130	2020	200	620	2840	580	140
Males†	26	6700	3650	450	1760	—	400‡	2160	240	160

*University students at Salt Lake City (elevation 5000).
†Volunteers at New Haven (sea level). Havens and associates: Am. J. M. Sc. 212: 129. 1946.
‡Classified as "Large Lymphocytes."

*These studies were performed with the cooperation of Dr. Gardner Middlebrook of the National Jewish Hospital at Denver, Colo.

Plate I.—*A*, Normal human lymphocyte. *B*, Type II lymphocyte (Downey) from a case of infectious mononucleosis. *C*, Type II lymphocyte from a normal subject given epinephrine. *D*, Monocyte. *E*, "Stress" lymphocytes from a stressed, adrenalectomized CBA mouse.

the Salt Lake City group are not striking, although the females tended to have slightly higher normal lymphocyte and slightly lower Type II lymphocyte levels. Although Havens classified lymphocytes as large and normals, both the description of the large lymphocytes and the plate presented in the manuscript coincide with the criteria employed here for Type II lymphocytes. The proportion of lymphocytes which are Type II was 27 per cent for the males and 18.3 per cent for the females of the Salt Lake group and 18.5 per cent for the males of the New Haven group.

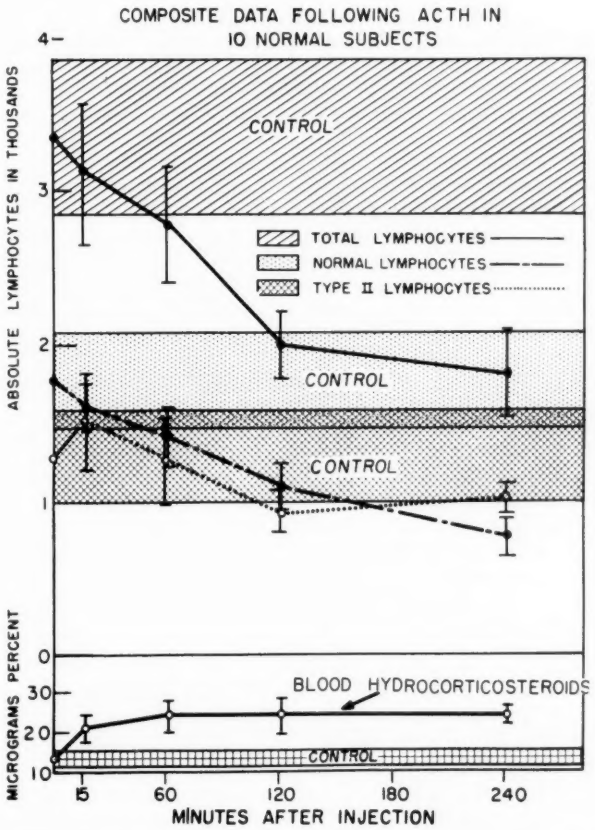

Fig. 1.—Absolute numbers of total, normal, and Type II lymphocytes and levels of blood 17-hydroxycorticosteroids following intravenous injection of 15 mg. ACTH in ten normal subjects.

Analysis of the lymphopenia induced by increased adrenocortical hormone secretions following injection of ACTH reveals that a significant lymphopenia was present at two and four hours. This lymphopenia was due to a decrease in numbers of normal lymphocytes. Numbers of Type II lymphocytes did not decrease significantly from the control levels. Blood 17-hydroxycorticosteroids were found to be elevated fifteen minutes after the injection of ACTH and remained at an increased level throughout the experimental

period (Fig. 1). In the ten subjects who received ACTH there was an inverse correlation between the level of blood 17-hydroxycorticosteroids and absolute numbers of normal lymphocytes. The numbers of Type II lymphocytes were not significantly altered by the elevation of the blood 17-hydroxycorticosteroids.

The composite data from two normal subjects who received epinephrine are presented in Fig. 2. The lymphocyte response differs from that following ACTH in that a lymphocytosis of both normal and Type II lymphocytes was found at one hour and persisted until the third hour. By the end of the eight-hour experimental period a lymphopenia was found which was due to

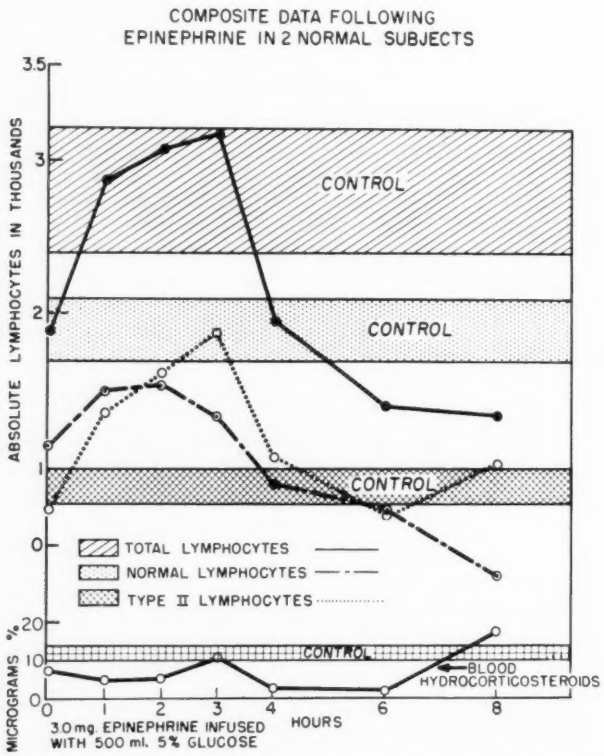

Fig. 2.—Absolute numbers of total, normal, and Type II lymphocytes and levels of blood 17-hydroxycorticosteroids following infusions of 3.0 mg. of epinephrine over a four-hour period in two normal subjects.

a decrease in numbers of normal lymphocytes. At four and six hours there was a decrease of the blood 17-hydroxycorticosteroid levels to extremely low values. However, these steroid levels were increased above the control value by the eighth hour. Again, as in the ACTH study, an inverse correlation between normal lymphocytes and blood 17-hydroxycorticosteroid levels was found (Fig. 2).

The effects of advanced cirrhosis on the lymphocyte response and blood levels of 17-hydroxycorticosteroids is seen in a six-day hospital course of a

33-year-old white man (Fig. 3). Three days prior to death the blood 17-hydroxycorticosteroids were found to be markedly elevated. No marked alteration of numbers of lymphocytes occurred until one day prior to death. The ensuing lymphopenia was initially one of normal lymphocytes, but eventually was due to a decrease in numbers of all lymphocytes. One hour prior to death normal lymphocytes had disappeared entirely. 80/c.mm. Type II lymphocytes remained as compared to an initial level of 720/c.mm.

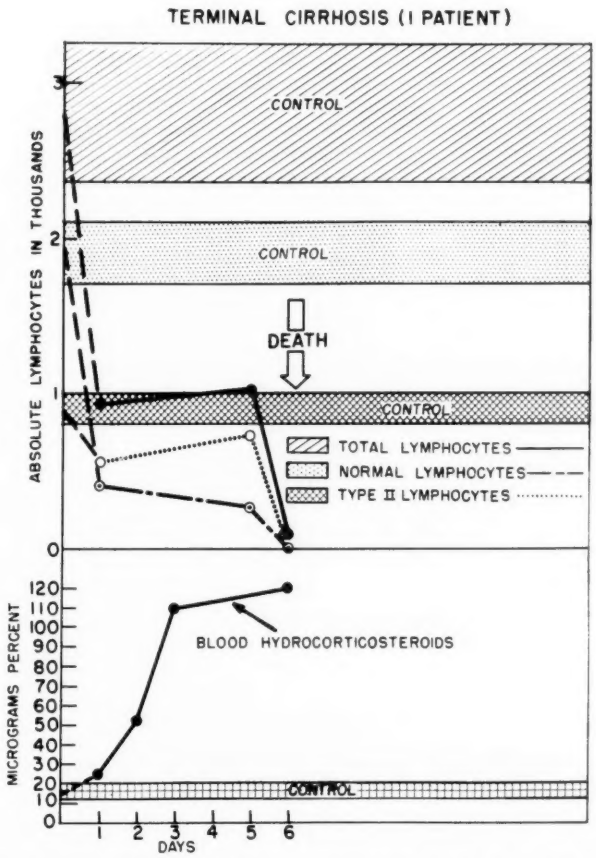

Fig. 3.—Absolute numbers of total, normal, and Type II lymphocytes and levels of blood 17-hydroxycorticosteroids following intravenous injection of 15 mg. ACTH in ten normal with advanced cirrhosis.

Type II lymphocytes. In the presence of extremely high levels of blood 17-hydroxycorticosteroids, normal lymphocytes were entirely absent. Following the loss of normal lymphocytes, Type II lymphocytes were also diminished in number.

The lymphocytosis of infectious mononucleosis (heterophil titer 1/448) in a 22-year-old white man was found to be due entirely to an increase in the number of Type II lymphocytes. A lymphopenia of normal lymphocytes was

present at the onset of the disease. Recovery was accompanied by a decrease in Type II lymphocytes and an increase in normal lymphocytes (Fig. 4).

Finally, a study was designed to test the value of the lymphocytic index (Type II/normal) as an indication of the clinical status of patients with tuberculosis. The lymphocytic indices of the group of normal subjects and the three groups of patients with tuberculosis are presented in Table II.

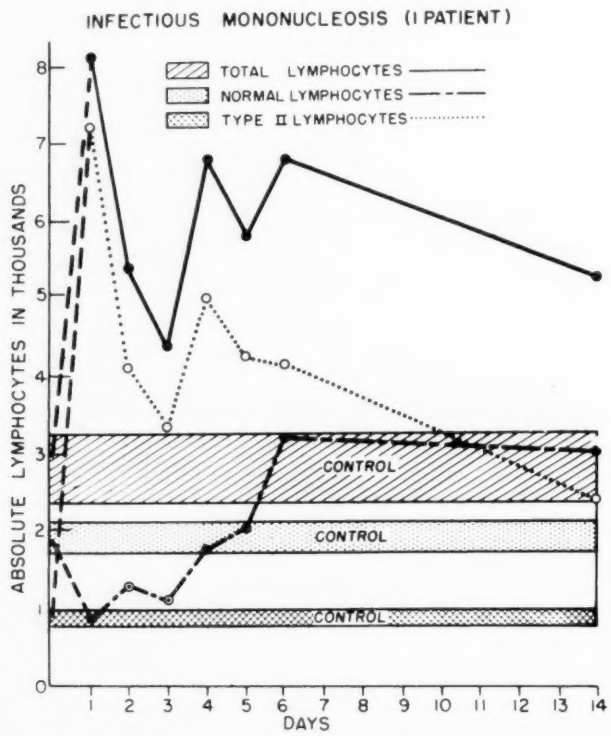

Fig. 4.—Absolute numbers of total, normal, and Type II lymphocytes during the course of infectious mononucleosis in a 22-year-old white man.

It can be seen that there was an excellent correlation between the hematologic and clinical (National Tuberculosis Association criteria) evaluations. The well group (lymphocytic index 0.33) corresponded hematologically with the controls, whereas both groups of patients having active tuberculosis had indices greater than the control groups.

DISCUSSION

Observations on the morphologic alterations of lymphocytes induced by stressors in adrenalectomized mice stimulated clinical investigation. The Type II lymphocyte as described here is morphologically identical with the stress lymphocytes of mice as demonstrated in a previous publication.

TABLE II. USE OF THE LYMPHOCYTIC INDICES (TYPE II/NORMAL) AS A METHOD FOR EVALUATION OF THE STATUS OF PATIENTS WITH TUBERCULOSIS

GROUP	NO. IN GROUP	LYMPHOCYTIC INDEX (TYPE II/NORMAL)	HEMATOLOGICAL EVALUATION	ACTUAL CLINICAL EVALUATION*
Control	54	0.36 ± 0.05	Well	— — — —
1	10	0.42 ± 0.04	Active—under treatment	Active—under treatment
2	10	0.33 ± 0.2	Well	Well
3	10	0.86 ± 0.14	Active—untreated	Active—untreated

*Criteria of the National Tuberculosis Association.

Type II lymphocytes were originally described by Downey, who called attention to the appearance of these cells in leukemoid reactions as opposed to the types of cells found in lymphatic leukemia.[17] In addition, he described Type I lymphocytes, primarily characterized by an increased amount of basophilic spongioplasm, and Type III lymphocytes similar to Type II lymphocytes with the exception that the cytoplasm is more vacuolated and basophilic. However, it has been pointed out by a host of investigators that morphologically altered lymphocytes thought to be pathognomonic of infectious mononucleosis occur in varying numbers both in normal subjects and in a wide variety of disease entities.[7-15] For example, because of the preponderance of these cells in virus diseases, Litwins and Leibowitz have proposed the name "virocytes."[7] Randolph has called attention to the possible diagnostic confusion between allergic diseases and infectious mononucleosis because of the presence of many "infectious mononucleosis" lymphocytes in the blood of allergic patients who exhibit no other laboratory or clinical signs of the disease.[8, 9] Also, there are reports of subclinical cases of infectious mononucleosis, i.e., atypical blood picture accompanied by no increase in heterophil titer nor diagnostic clinical signs.[10, 18] Havens reported increases in numbers of Type II lymphocytes following the experimental induction of infectious hepatitis.[11]

The widespread recognition that the "infectious mononucleosis" lymphocyte is not specific for infectious mononucleosis is further confirmed by observations in this laboratory that significant numbers of Type II lymphocytes are present in blood from patients with rheumatoid arthritis, acute anxiety neurosis, bronchial asthma, Addison's disease, lupus erythematosis, chronic infections, rheumatic fever, infectious lymphocytosis, infectious hepatitis, and patients undergoing elective surgery. In addition, Ingram and coworkers have found lymphocytes of this type as well as lymphocytes with bilobed nuclei in the blood of cyclotron workers, but state that they are not specific for radiation but occur in numerous disease entities.[13]

Experience in this laboratory has been that Type I lymphocytes occur in small numbers and do not vary appreciably. Consequently, they have been added to the total number of normal lymphocytes. For all practical purposes, the differences between Type III lymphocytes and Type II lymphocytes are sufficiently insignificant that they can be classified together. The morphologic

differences between the three types of lymphocytes do exist, but a more complex classification does not contribute appreciably to an analysis of the stress lymphocyte response.

The number of lymphocytes present in normal males and females in Salt Lake City (elevation 5,000 ft.) is higher than that found by Havens at sea level.[11] The increased number of lymphocytes at higher altitudes has been noted previously.[19] Regardless of differences in total numbers of lymphocytes at these two altitudes, the proportion of Type II lymphocytes is similar in both groups.

It is apparent that there is a differential susceptibility of normal and Type II lymphocytes to the lytic effects of adrenocortical hormones. In the presence of increased blood levels of 17-hydroxycorticosteroids a lymphopenia of normal lymphocytes occurred in the ten subjects who received ACTH, in the two epinephrine-treated subjects by the eighth hour when the 17-hydroxycorticosteroids had increased, and, although 17-hydroxycorticosteroid levels were not measured in the case of infectious mononucleosis, it can be inferred that the decrease in numbers of normal lymphocytes was due to increased adrenocortical secretion. That the stress lymphocytes are not entirely resistant to destruction is evidenced in the case of advanced cirrhosis in which the level of Type II lymphocytes decreased from 720 per cubic millimeter to 80 per cubic millimeter. This differential susceptibility of lymphocytes is further substantiated by observations in this laboratory which demonstrate a decrease in numbers of normal lymphocytes and not of stress lymphocytes in stressed, intact mice.[5] The adrenocortically mediated lymphopenia of normal lymphocytes occurred two to four hours following elevation of 17-hydroxycorticosteroids in the blood, and confirms the initial findings of Nelson and Sandberg who demonstrated a 17-hydroxycorticosteroid peak at one hour following oral administration of Compound F acetate and a maximum decrease of lymphocytes at four hours when the steroid level had fallen.[20]

The functional capacity of the adrenal cortex is dependent upon the rate of secretion of adrenocortical hormones and the rate of requirement for these hormones imposed by the tissues. Measurements of the amounts of hormone secreted into the blood do not indicate whether or not the amount of hormone secreted is sufficient to meet the optimum hormonal requirements of the tissues. This information could be obtained provided the effects of the hormone on a particular tissue were delicate and quantitative enough to provide an index of rates of secretion and rates of requirement. An ideal situation would exist provided we were able to measure the amount of adrenocortical hormone secreted per unit of time and the rate at which it is removed from the blood to take part in a specific, measurable process. Although ACTH or some conditions of stress will raise the concentration of adrenocortical hormone as measured by Nelson,[20] such measurements give no indication of the rate of requirement. It appears that one of the key assumptions concerning adrenocortical physiology is that graded degrees of

requirement are imposed by graded stimuli. This point of view has not been emphasized sufficiently, in spite of the evidence obtained from the commercial use of the Ingle muscle fatigue test[21] in addition to work in our own laboratory on resistance to anaphylactic shock, histamine poisoning, inflammatory response, bacterial pyrogens, irradiation, epinephrine, and the graded lymphocytolytic effects of various adrenocortical hormones.

Previous investigations on the lymphocyte response of stressed, adrenalectomized mice have shown that the lymphocytosis following stress is due primarily to an increase in numbers of stress lymphocytes. This has been demonstrated using epinephrine and a wide variety of stressors. Large amounts of epinephrine such as the 3.0 mg. dose employed here resulted in a diminution of blood 17-hydroxycorticosteroids to extremely low values. It has been suggested by Dougherty,[22] and recently reviewed by Ingle[23] that increased amounts of stress result in increased "utilization" of hormone.

It is apparent from both the epinephrine and ACTH data presented here that the numerical response of lymphocytes reflects and anticipates changing blood levels of 17-hydroxycorticosteroids, and that the number of circulating lymphocytes vary inversely with the amount of adrenocortical hormone in the blood. This is further substantiated by the lymphopenia observed in the case of advanced cirrhosis. The increase in numbers of stress lymphocytes in infectious mononucleosis can be related to experiments on stressed, adrenalectomized mice in which large numbers of stress lymphocytes are produced. Therefore, it is postulated that an increase in numbers of stress lymphocytes is not solely related to insufficient amounts of available adrenocortical hormone, but that another factor not mediated by the adrenal cortex is necessary for their production (*an*-adrenocortical mediated stress response).

The data presented here strongly suggest that the numbers and types of circulating lymphocytes reflect the degree of balance existing between adrenocortically mediated and nonadrenocortically mediated responses to stress stimuli. This hypothesis is strengthened by the finding that normal lymphocytes decrease in number following an increase of blood 17-hydroxycorticosteroids in the ACTH-treated group and that Type II lymphocytes increase in number when there is a drop in blood levels of 17-hydroxycorticosteroids as seen in the epinephrine-treated group. The differential susceptibility of normal and Type II lymphocytes to the presence of high blood levels of 17-hydroxycorticosteroids is also demonstrated in the case of advanced cirrhosis in that normal lymphocytes disappeared from the blood earlier than the Type II lymphocytes.

The accurate correlation of the ratio of Type II/normal lymphocytes with the clinical evaluation of patients with tuberculosis suggests further trial of this sensitive measurement in this and other disease entities.

These clinical cases were selected in order to evaluate differences that exist at the lymphocytic level with varying amounts of adrenocortical hormones and varying degrees of stress. Animal and clinical studies indicate

the presence of an as yet unknown factor which produces morphologically altered lymphocytes ("stress" lymphocytes) under conditions of stress not mediated by adrenocortical secretions. Evidence at hand using growth hormone and thyroxin gives no indication that either of these hormones can account for the marked increase in number of this type of circulating lymphocyte following stressors.[24]

SUMMARY

The quantitative and qualitative changes of blood lymphocytes of normal subjects given ACTH, normal subjects given epinephrine, and a patient with advanced cirrhosis were assessed and correlated with blood levels of 17-hydroxycorticosteroids.

The lymphocytic changes accompanying infectious mononucleosis, tuberculosis, and a wide variety of disease entities were studied.

Lymphocytes were classified as normal and Type II lymphocytes (stress lymphocytes). The ratio of Type II/normal lymphocytes was designated as the lymphocytic index, assuming that an increased index (more stress lymphocytes, fewer normal lymphocytes, or a combination of both) reflected greater stress.

Type II lymphocytes are not specific for infectious mononucleosis but occur in blood of normal subjects and various types of diseases.

Type II lymphocytes appear to be less susceptible to the lytic effects of adrenocortical hormones than normal lymphocytes. It is suggested that Type II lymphocyts are produced by a nonadrenocortically mediated response to stress.

The numerical response of lymphocytes reflects and anticipates changing blood levels of 17-hydroxycorticosteroids and varies inversely with the amount of adrenocortical hormone in the blood.

The numbers and types of circulating lymphocytes reflect the degree of balance existing between adrenocortically mediated and nonadrenocortically mediated responses to stress stimuli.

The accurate correlation of the lymphocytic index with the clinical evaluation of patients with tuberculosis suggests further trial of this sensitive measurement in this and other disease entities.

The authors wish to thank Duwayne Schmidt for his assistance in obtaining material.

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ETHYLENEDIAMINETETRAACETIC ACID IN THE MOBILIZATION AND REMOVAL OF IRON IN A CASE OF HEMOCHROMATOSIS

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HEMOCHROMATOSIS is a disease characterized, in part, by deposits of large amounts of iron-containing pigment throughout the body, with particular concentration in the liver, spleen, and pancreas. Since the human body has little capacity for the excretion of iron, the use of phlebotomy to remove iron stores from the body has thus far proved the only useful tool for the mobilization of the excess iron. Davis and Arrowsmith[1] have reported that in several cases of hemochromatosis, using repeated phlebotomies, there were no untoward effects in any of the cases. However, in cases of advanced hemochromatosis where there is hepatic and pancreatic involvement, it has been suggested that this procedure may not be advisable.[2] The strain on the hematopoietic system from frequent phlebotomies is also not desirable. A method for the removal of excess iron, other than that of phlebotomy, would, therefore, be preferable, particularly in most cases of hemochromatosis.

The ability of certain organic compounds to form strong nonionic water-soluble combinations with metal ions in biological systems suggests the possibility that these compounds might be useful in the removal of undesirable metal ions from the body. In this respect, Kety[3] has shown that citric acid may play a role in the removal of lead from bones by such a mechanism. A significant advance in the control of metal ions in biological systems has been reported by Popovici, Geschickter, Reinovsky, and Rubin,[4] who regulated serum calcium levels in vivo by use of ethylenediaminetetraacetic acid (EDTA). Foreman and Hamilton[5] demonstrated that EDTA will chelate plutonium and yttrium in vivo and thereby greatly accelerate their excretion from the body. Bessman, Reid and Rubin[6] used the disodium calcium versenate* for the removal of lead in vivo with excellent results. Foreman and associates[7] used "Fe—3 Specific" to accelerate the excretion of radioactive iron from rats.

This report deals with the application of this principle for increasing iron excretion in a case of hemochromatosis. The chemical chosen for this study was disodium calcium versenate.†

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*The disodium calcium salt of ethylenediaminetetraacetic acid is also known as disodium calcium versenate.
†We wish to thank the Riker Laboratories, Los Angeles, Calif., for kindly providing the material used in this study.

CASE REPORT

N. L., a 54-year-old Italian cook, was admitted to the private Medical Service of the Sinai Hospital (Dr. B. B.) on March 17, 1952, with the history of gas and belching of two months' duration. The patient had seen a physician who told him that "something was wrong with his liver" and put him on a diet which restricted the use of "greasy and fatty foods." He had been a heavy drinker for many years, but in the five years prior to admission had drunk only on week ends.

Physical examination on this admission revealed a tall, stocky man with a slightly yellowish-brown tint to the skin. The blood pressure was 140/90. The liver edge was palpable 13 cm. below the right costal margin. The spleen was palpable 4 cm. below the left costal margin. There was a small umbilical hernia.

The laboratory findings were as follows: serological test for syphilis, negative; blood sugar, 105 mg. per cent; blood urea, 42 mg. per cent; hematocrit, 49; sedimentation rate (corrected) 2. The white blood cell count was 7,500 per cubic millimeter with a differential of 56 per cent polymorphonuclears and 44 cent lymphocytes; platelets 86,000 (repeat 76,000); bleeding time three minutes; clotting time nine minutes; prothrombin activity 100 per cent. The urine had a specific gravity of 1.030, showed a trace of albumin, and was negative for sugar and acetone. Bromsulfalein retention was 26.8 per cent in thirty minutes. The cephalin flocculation was 3 plus in forty-eight hours. The thymol turbidity was 5.4 units; alkaline phosphatase 4.6 units; albumin 4.9 Gm. per cent; globulin 1.7 Gm. per cent. The indirect Van den Bergh was 1.3 mg. per cent; the direct Van den Bergh 0.2 mg. per cent; blood cholesterol 177 mg. per cent.

The patient had no evidence of diabetes on this admission.

The patient was discharged from the hospital three days after admission, and did well until September, 1952, when he began noting dryness of the mouth, polydypsia, polyuria, and weakness of the legs. He consulted his physician who found his blood sugar elevated and glycosuria present. The patient was put on a diabetic diet and protamine zinc insulin. The diabetes was difficult to control and the insulin dosage was constantly readjusted, the patient being finally controlled on protamine zinc insulin and regular insulin. The second week in December, 1952, the patient developed drowsiness, the diabetes again became difficult to control, and he was readmitted on Jan. 3, 1953.

Physical examination on this admission revealed that the skin had a slightly brownish tint, especially over the tibias and over the forearms and dorsa of the hands. There was scanty hair on the chest and abdomen and scanty axillary hair. The voice was not particularly high pitched. The testicles were found to be quite reduced in size and soft, particularly the right one. The liver margin was palpable just above the iliac crest, and was nodular and firm. The spleen was palpable 6 cm. below the left costal margin.

The laboratory findings were as follows: white blood cell count 7,500 per cubic millimeter with a differential of 48 per cent polymorphonuclears, 40 per cent lymphocytes, 9 per cent monocytes, and 3 per cent eosinophils; platelets 106,000 (repeated 149,000); hematocrit 45; hemoglobin, 12.1 Gm. per cent; bleeding time two minutes; clotting time nine minutes; prothrombin activity 85 per cent. The blood sugar was 380 mg. per cent; serum calcium 11.1 mg. per cent; serum phosphorus 4.5 mg. per cent; blood serum sodium 140 meq. per liter; serum potassium 5.7 meq. per liter; albumin 5.4 Gm. per cent; globulin 2.0 Gm. per cent; thymol turbidity 7.2 units; indirect Van den Bergh 0.4 mg. per cent; direct Van den Bergh 2.1 mg. per cent.

On the days following admission the patient's blood sugar varied as follows: 470, 333, 198, 385, and 161 mg. per cent. The diabetes was controlled with difficulty, and the insulin dosage was increased from 60 units of protamine zinc insulin and 20 units of regular insulin to 70 units of protamine zinc insulin and 35 units of regular insulin every morning. Control was ultimately achieved with 60 units of protamine zinc insulin and 30 units of regular insulin every morning.

At this time an aspiration biopsy of the liver was performed.

Histologic examination of the aspirated material revealed a core of liver tissue which showed complete distortion of the lobular achitecture with the formation of nodules of liver cells and other features usually seen in cases of Laennec's cirrhosis. However, in addition, there were demonstrable striking deposits of iron pigment, both in the Kupfer cells and in the parenchymal cells of the liver. There were also seen within the parenchymal cells scattered aggregates of a nonferrous pigment shown to be hemofuscin. A *diagnosis* was made of cirrhosis of the liver as seen in cases of hemochromatosis.

METHODS AND MATERIAL

The study extended over a thirteen-day period with the first five days being used as the control period, the sixth through the tenth day being the experimental period, and the eleventh through the thirteenth day being used as another control period.

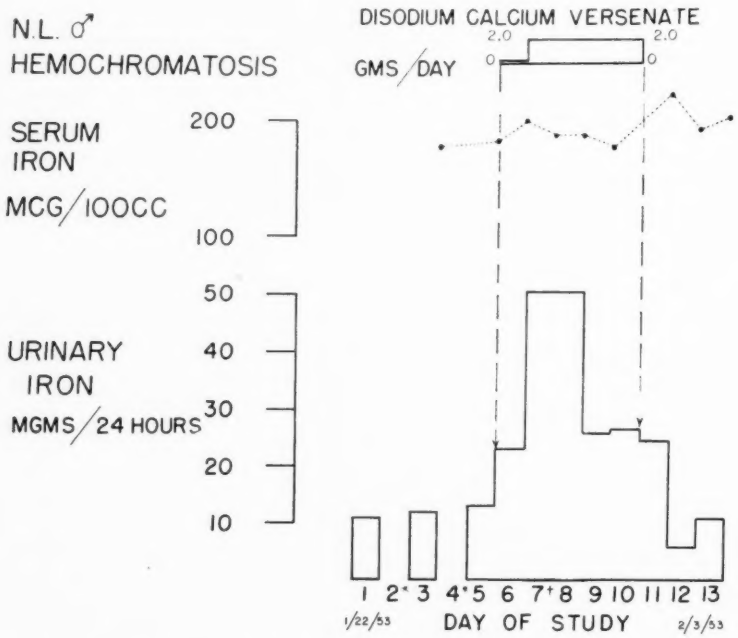

Fig. 1.—Effect of disodium calcium versenate on urinary iron excretion and serum iron levels.
*Faulty urine collection; specimens discarded.
†Specimens for days 7 and 8 were combined in error; a mean value is shown.

A test dose of 0.2 Gm. of disodium calcium versenate in 250 ml. of 0.9 per cent saline was administered intravenously over a one-hour period on the sixth day. No toxic reaction manifested itself. For the following four days, one gram of disodium calcium versenate in 250 ml. of 0.9 per cent saline was administered intravenously over a one-hour period twice daily.*

Twenty-four hour urine specimens were collected daily, the volume recorded and the samples refrigerated until the iron determinations were made. Fasting samples of blood were drawn for serum iron determinations.

Care was taken that all apparatus and reagents used in collection, storing, or analysis of specimens were iron-free.

*These dosages were based on recommendations of Dr. Norman W. Karr, Director of Clinical Research, Riker Laboratories Inc., Los Angeles, Calif.

Serum iron was determined by the method of Kitzes, Elvehjem, and Scheutte.[8] Samples were analyzed in duplicate and recovery of added iron to serum showed a ±5 per cent error in the method. Urine iron content was determined by the method of Elvehjem.[9] Triplicate determinations were made, and recovery procedures gave an average error of ±5 per cent. All other laboratory tests were performed using standard procedures.

RESULTS AND DISCUSSION

As shown in Fig. 1, during the control period the patient excreted approximately 1.2 mg. of iron per day. This is almost four times the normal average daily value reported by other workers.[10]

During the four-day period of calcium disodium versenate administration, the iron excretion was 15 mg., or 3.75 mg. per day. This represents an increase of more than three times the amount excreted daily during the control period. This elevated iron excretion continued for one day after withdrawal of the drug, but was at the control level when the study ended.

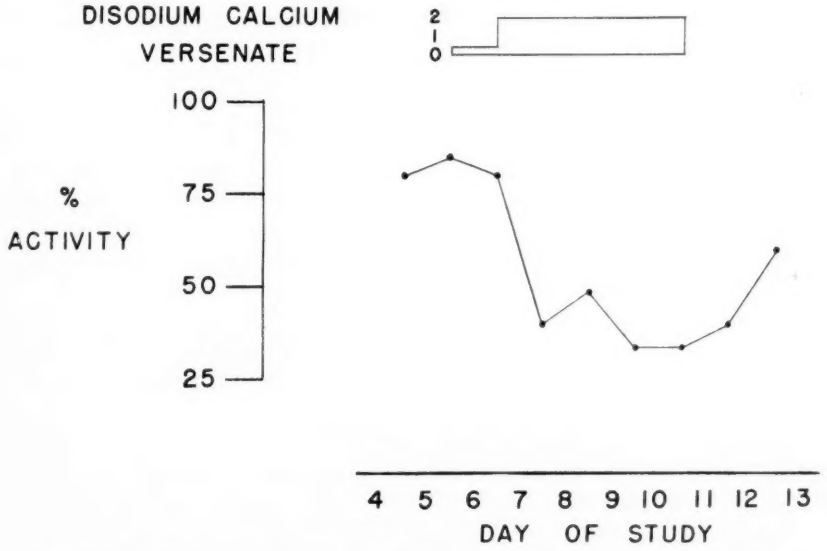

Fig. 2.—Effect of disodium calcium versenate therapy on prothrombin activity.

It is to be noted that the relatively small test dose of the experimental drug was sufficient to raise the patient's urinary iron excretion to twice his control level. The further increase after initiation of the daily administration of 2 Gm. was most marked on the first two days of the experimental period. With the continued administration of the disodium calcium versenate, iron excretion fell to a level higher than control values, but equivalent to that following the initial test dose. All trends observed in the iron values obtained are those which lie outside the experimental error of the procedures used.

Serum iron values are shown in Fig. 1. The values obtained are consistent with the high serum iron concentration reported in cases of hemochromatosis.[11] At present, we have no explanation for the changing serum iron levels.

During the four-day administration of the experimental drug 10.2 mg. of iron in excess of average normal excretion was removed. The amount of iron that would be lost if 100 ml. of whole blood were removed from a patient would be approximately 50 mg. It is obvious that the iron removed by the compound chosen was not sufficiently great to consider this procedure a substitute for the method of phlebotomy. However, the use of a chemical to remove iron from the body appears to be feasible. Choice of another chelating compound may ultimately yield a new treatment for the disorder.

The patient's condition remained generally unchanged during the study. Daily laboratory determinations of blood sugar, urea, sodium, potassium, calcium, phosphorus, bilirubin, hemoglobin, hematocrit, white blood cell count, differential count, and platelet determinations showed no significant change. Liver function tests, clotting time, clot retraction, and bleeding time determinations made before and after the study showed no variation. Prothrombin activity was determined daily and values obtained are shown in Fig. 2. These values declined rapidly after administration of the drug, despite Vitamin K therapy during the entire course of study.

SUMMARY

Disodium calcium versenate has been used to effect mobilization and removal of excess iron stores in a case of hemochromatosis.

A threefold increase of urinary iron was observed during administration of the drug.

A rapid fall in the patient's prothrombin activity was noted as the only untoward effect of the procedure.

We wish to acknowledge the assistance of Miss Marcella Shemer in performing the prothrombin activity determinations reported in this study.

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IMMUNOLOGIC MECHANISMS OF LEUKOCYTE ABNORMALITIES

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IMMUNOLOGIC mechanisms are responsible for certain disorders involving the erythrocytes (e.g., acquired hemolytic anemia) and platelets (e.g., thrombocytopenic purpura). It is probable that similar mechanisms may be responsible for diseases of the leukocytes, although definite clinical and laboratory data to corroborate this probability are sparse. There is well-documented evidence as early as 1907 that in certain human leukocytic diseases a plasma factor could be demonstrated which was capable of producing phagocytosis, clumping, and lysis of leukocytes.[1-3] More recently, similar phenomena have been observed in disseminated lupus erythematosus,[4] and subsequent studies have shown that this is due to an abnormal component of the plasma gamma globulin.[5, 6] In certain cases of agranulocytosis and drug-induced leukopenia, a plasma factor thought to be leukocyte antibody has been reported.[7-13]

The purpose of this report is to present observations on the action of an experimentally produced leukocyte antiserum on human leukocytes. Agglutination, lytic change, and phagocytosis of leukocytes developed in a manner very similar to that observed in disseminated lupus erythematosus and certain human leukopenic states. These observations suggest that the plasma factor present in disseminated lupus erythematosus and in certain other leukopenic disorders may be a leukocyte antibody, and that the "L. E. phenomenon" is a manifestation of a leukocyte antigen-antibody response.

EXPERIMENTAL PROCEDURE

A. *Preparation of Leukocytic Antiserum.*—Fifty to one hundred milliliters of venous blood was collected through a resin* column into sterile polyvinyl plastic systems from patients with chronic granulocytic and chronic lymphocytic leukemias. The total leukocyte counts ranged from 170,000 cells per cubic millimeter to 360,000 cells per cubic millimeter. These whole blood samples were sedimented by gravity for one hour; the supernatant plasma leukocyte suspensions, containing from 50 to 100 leukocytes per erythrocyte, were removed and the leukocytes were washed three times with sterile physiologic saline. Each collection of leukocytes was incorporated into a modified Freund's adjuvant[14] and for three consecutive weeks each of three albino rabbits was injected subcutaneously with one milliliter of the leukocyte-adjuvant mixture. Five weeks after the initial injection each animal was injected subcutaneously with one milliliter of sterile saline-washed leukocytes obtained from the same patients. On the sixth week thirty milliliters of blood was removed from each rabbit by cardiac puncture; the serum was separated, and stored at −75° C. Each lot of rabbit

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This study was presented in part at the annual meetings of the Eastern Section of the American Federation for Clinical Research in New York City on Dec. 5, 1952, and the American Society for Clinical Investigation at Atlantic City, N. J., May 4, 1953.

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*Dowex 50.

serum then was absorbed three times with equal volumes of packed types AB and O saline-washed human erythrocytes in order to remove antierythrocyte and nonspecific antihuman components. The serum so prepared was designated as "antileukocytic serum."

B. *Action of Antileukocytic Serum.*—

1. *In vitro cytologic studies:* Plasma-leukocyte suspensions were obtained from normal subjects and patients with a variety of illnesses by allowing the erythrocytes of their heparinized venous bloods to sediment by gravity at room temperature. To 1.0 ml. aliquots of these plasma-leukocyte suspensions, from 2 to 4 drops of antileukocytic serum was added, mixed, and incubated at 37° C. for thirty minutes. Normal rabbit serum, Coombs' serum, and L. E. serum added to other aliquots of the plasma-leukocyte suspension were used for control. Other preparations were made in a similar fashion, using heat-inactivated (56° C. for thirty minutes) antileukocytic serum incubated with either fresh plasma-leukocyte suspensions or saline-washed leukocytes which were resuspended in their own heat-inactivated plasma. Portions of many of these mixtures were observed directly as wet preparations under oil immersion magnification during the period of incubation. Following incubation each mixture was centrifuged at 100 g for two to three minutes, and coverslip smears of the sedimented leukocytes were made and stained with Wright's stain.

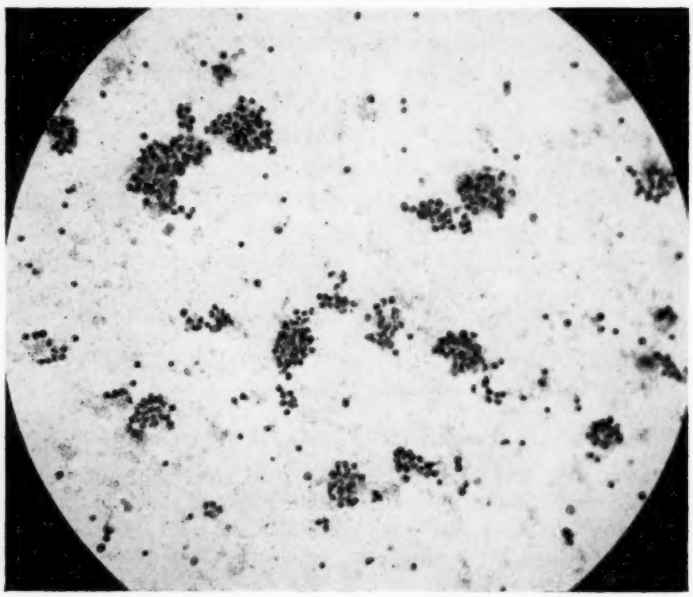

Fig. 1.—Clumping of normal polymorphonuclear leukocytes following incubation with antigranulocytic serum. (×230, reduced ⅓.)

2. *Microscopic leukoagglutination studies:* Separate serial saline dilutions of the antileukocytic serum and normal rabbit serum were made and to each dilution an equal volume of human plasma containing either granulocytic or lymphocytic leukemic leukocytes, adjusted by dilution with saline to a concentration of 50,000 cells per cubic millimeter, was added. After thirty minutes' incubation at room temperature (20° C. to 23° C.) wet cover-slip preparations of each suspension were examined under the microscope for evidence of agglutination.

3. *Macroscopic leukoagglutination studies:* To one milliliter of plasma containing human leukemic leukocytes, adjusted to a concentration of 50,000 cells per cubic millimeter with physiologic saline, two to four drops of either normal rabbit serum or antileukocytic serum was added. These were transferred immediately to Wintrobe hematocrit tubes and were allowed to sediment by gravity for periods ranging up to four hours. At twenty-minute intervals the height of each column of sedimented leukocytes was observed.

Fig. 2.

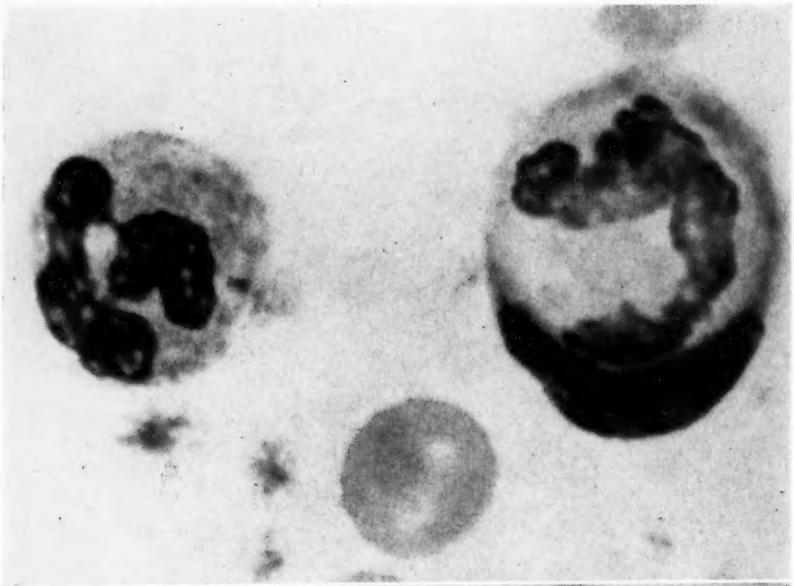

Fig. 3.

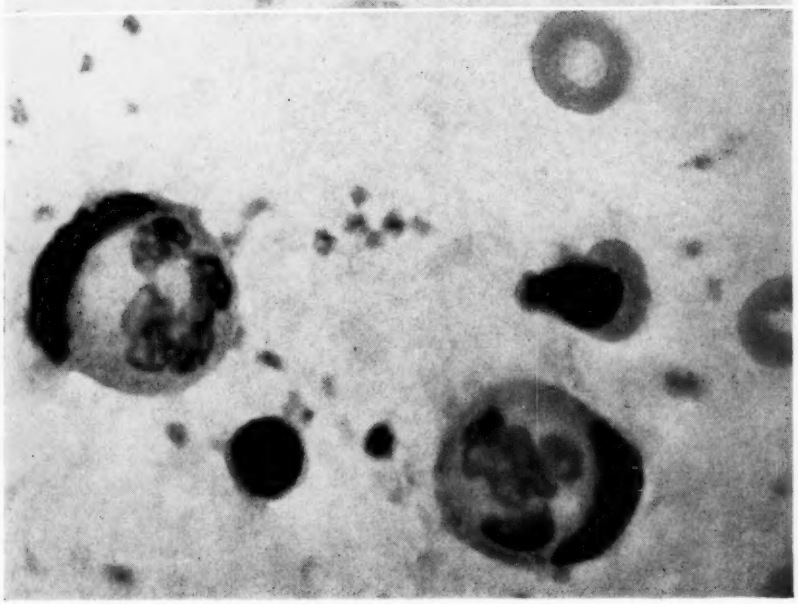

Fig. 2.—Single polymorphonuclear leukocyte completely phagocytized by a monocyte following incubation of normal leukocyte with anti-granulocytic serum. (×6,000, reduced 1/5.)

Fig. 3.—Two polymorphonuclear leukocytes, each completely engulfed by a monocyte. (×3,700, reduced 1/5.)

OBSERVATIONS

Following the incubation of human leukocytes with leukocyte antiserum, three striking morphologic features were observed consistently on the stained smear preparations: (1) clumping of granulocytes, (2) phagocytosis of granulocytes and (3) vacuolization of neutrophilic granulocytes and monocytes.

Discrete aggregates of granulocytes composed of from a few to 40 or 50 cells appeared in Wright's stained preparations following incubation of human leukocytes with the antigranulocytic serum, and to a much lesser extent with the antilymphocytic serum. These leukocyte clusters frequently resembled the "rosettes" of the L. E. test (Fig. 1), but often cell distribution was not uniform, and usually there was no central mass of amorphous debris present.

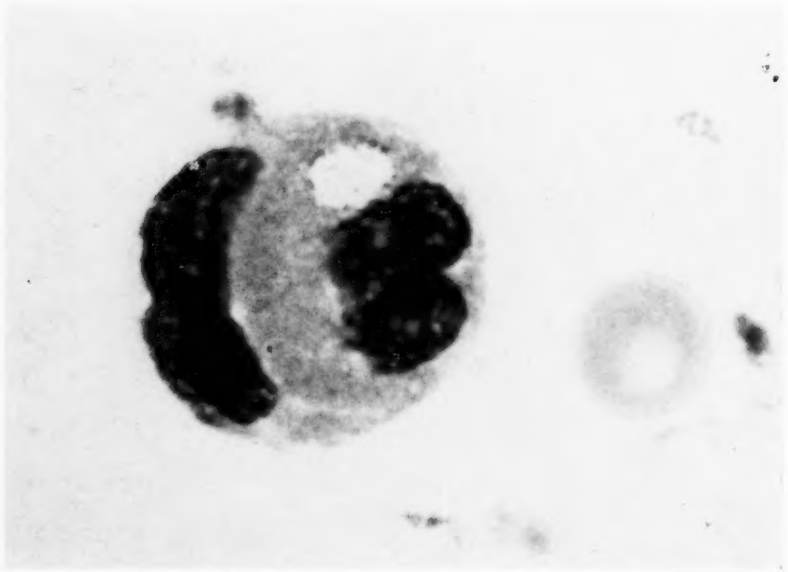

Fig. 4.—Eosinophilic leukocyte within a monocyte. (×6,000, reduced ⅓.)

The second unusual feature of these stained coverslip preparations was the presence of numerous leukocytes, each containing a single intracellular inclusion. These inclusions consisted of either a neutrophilic granulocyte, an eosinophilic granulocyte, or a homogenous purplish-blue or deep blue amorphous mass. In most instances the phagocytic cell had a deep blue, eccentrically placed, crescent-shaped nucleus which partially surrounded the cytoplasmic inclusion, but in a few there was definite nuclear lobulation. Most of the phagocytic cells contained an intact neutrophilic granulocyte which was surrounded by the deep blue nucleus and a rim of light blue cytoplasm (Figs. 2 and 3), but a few contained an eosinophilic granulocyte (Fig. 4). However, others contained a deep purplish-blue cytoplasmic inclusion, and presented an appearance indistinguishable from Hargrave's L. E. cell (Figs. 5 and 6). The nucleus of some of these L. E. type cells was lobulated and appeared to be that

Fig. 5.

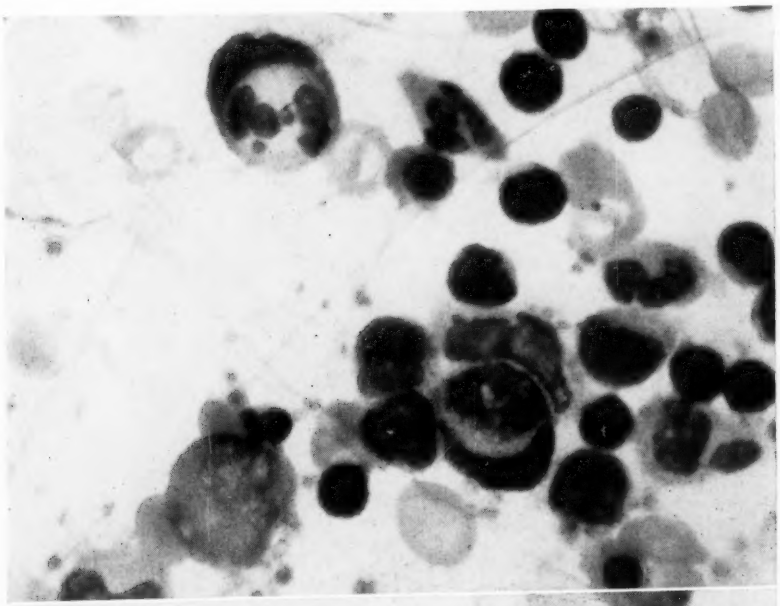

Fig. 6.

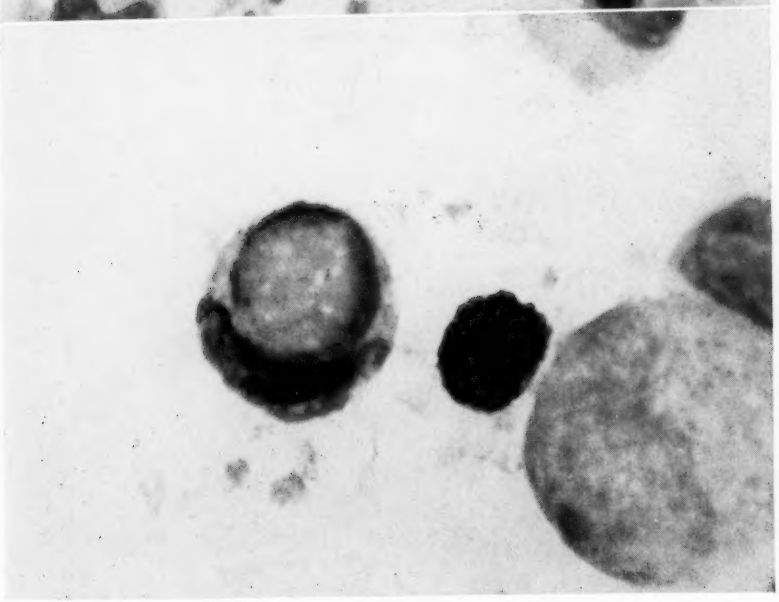

Fig. 5.—Amorphous inclusion in the cytoplasm of a monocyte appearing similar to the L. E. cell. (×3,700, reduced ⅓.)

Fig. 6.—Phagocytized polymorphonuclear leukocyte and L. E. type cell in the same field. (×1,800, reduced ⅓.)

Fig. 7.

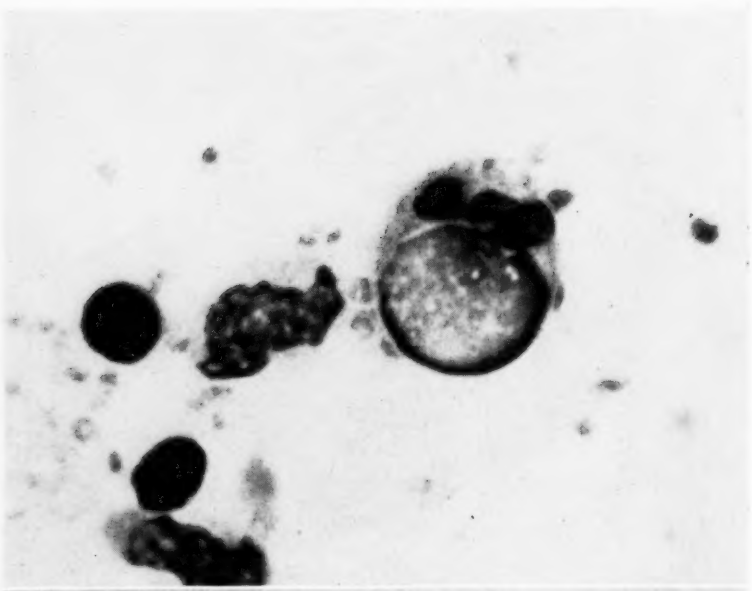

Fig. 8.

Fig. 7.—L. E. type cell with nuclear lobulation and characteristics of a polymorphonuclear leukocyte. (×3,700, reduced ⅓.)

Fig. 8.—Early stage of leukophagocytosis. The cytoplasm of a polymorphonuclear leukocyte is protruding into a vacuolated monocyte whose nucleus is assuming an eccentric position. (×3,700, reduced ⅓.)

Fig. 9.

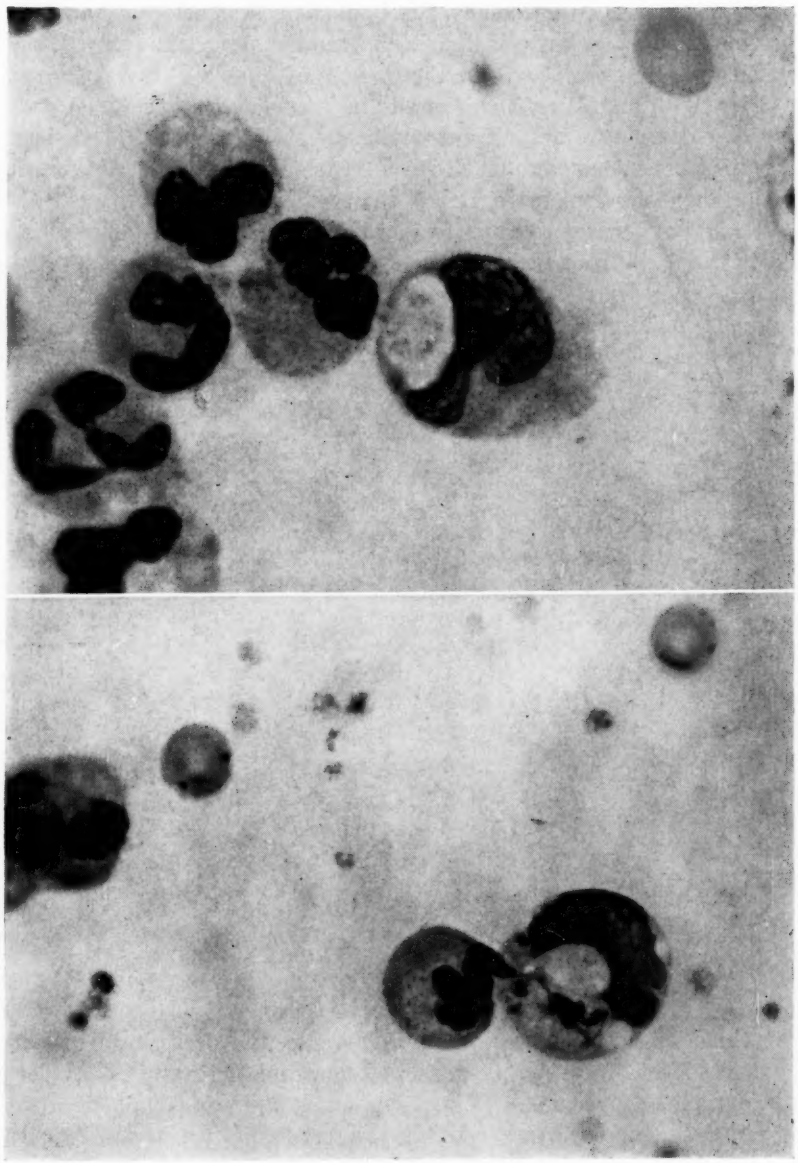

Fig. 10.

Fig. 9.—More advanced phase of leukophagocytosis. The cytoplasm of a monocyte contains a single large vacuole into which a polymorphonuclear leukocyte is entering. (×3,700, reduced ⅓.)

Fig. 10.—Filamented nucleus of a polymorphonuclear leukocyte preceding the entry of the entire cell into the vacuolated cytoplasm of a monocyte. (×2,000, reduced ⅓.)

of a polymorphonuclear leukocyte (Fig. 7). The identity of the phagocytic cell often was difficult to ascertain with certainty, but the finding of intermediate and early phases indicated that monocytes as well as neutrophilic granulocytes were phagocytizing. In some instances, a large mononuclear cell was observed with a neutrophilic granulocyte in the process of entering its vacuolated cytoplasm (Figs. 8, 9, and 10). Occasionally, a vacuolated monocyte containing a partially digested neutrophilic granulocyte was observed (Fig. 11). These leukophagocytic phenomena were much more frequently observed following incubation with the antigranulocytic serum than with the antilymphocytic serum. Phagocytic activity was not reduced when heat-inactivated antileukocytic serum was incubated with fresh plasma leukocyte suspensions, but leukophagocytosis was absent when heat-inactivated antileukocytic serum was incubated with saline-washed leukocytes in heat-inactivated plasma.

Fig. 11.—Distorted and partially digested polymorphonuclear leukocyte within the vacuolated cytoplasm of a monocyte. (×6,000, reduced ⅓.)

The third feature of these preparations was the occurrence of leukocyte lytic change. Most of the nonphagocytized neutrophilic granulocytes developed multiple cytoplasmic vacuoles (Fig. 12), but lymphocytes appeared morphologically intact. The antigranulocytic serum exerted much more effect on these cells than did the antilymphocytic serum.

Agglutination studies by either the sedimentation or tube agglutination techniques indicated that the antigranulocytic serum produced marked agglutination of human leukemic granulocytes but not lymphocytes. The antilymphocytic serum, on the other hand, had a very limited capacity to promote agglutination of either human leukemic granulocytes or lymphocytes. Absorption of either serum with human saline-washed leukemic granulocytes markedly re-

duced or removed its capacity to agglutinate leukocytes while absorption with human saline-washed lymphocytic leukemic cells was without effect. Although normal undiluted rabbit serum exhibited some leukocyte agglutinating ability in low titers, it did not approach the magnitude of response observed with the antigranulocytic serum. The results of microscopic leukocyte agglutination studies are presented in Table I, while those of the macroscopic sedimentation technique are shown in Fig. 13.

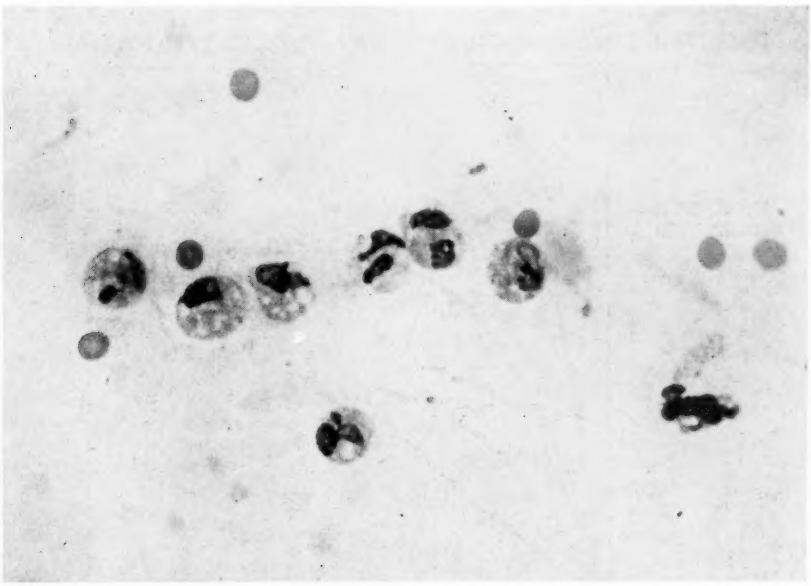

Fig. 12.—Multiple cytoplasmic vacuoles, distortion of cell outlines and nuclear degeneration of polymorphonuclear leukocytes following one-hour incubation with antigranulocytic serum. (×1,200, reduced ⅓.)

Although leukolytic changes invariably were observed, by no method have we been able to demonstrate complete dissolution of leukocytes following exposure to these antileukocytic sera. These methods have included warm stage direct microscopy, leukocyte counting, and determination of leukocyte hematocrit of leukemic leukocytes before and after incubation of human leukocyte suspensions with these rabbit sera.

Repeated in vitro observations with either normal rabbit serum or Coombs' serum mixed with human leukocytes have not produced evidence of leukophagocytosis, lysis, or any significant degree of leukoagglutination. On the other hand, when human L. E. serum was incubated with normal leukocytes these changes frequently were observed, and occasionally the phagocytosis of an intact polymorphonuclear leukocyte was seen (Fig. 14). In a similar manner the leukocyte antiserum has induced leukophagocytosis, leukoagglutination, and lytic change in leukocytes obtained from twenty-five consecutive patients, irrespective of age, sex, major blood group, or type of illness. The ability of both the antigranulocytic and antilymphocytic sera to produce these effects was re-

duced markedly or destroyed completely following absorption with granulocytic leukocytes, but was unchanged following absorption with lymphocytes. Leukophagocytic activity was not reduced by heating the antileukocytic serum to 56° C. for thirty minutes; the reaction did not occur below 10° C. or above 40° C., but proceeded rapidly at room temperature and at 37° C. over a pH range of from 7 to 8. Leukophagocytosis was observed when either small quantities of

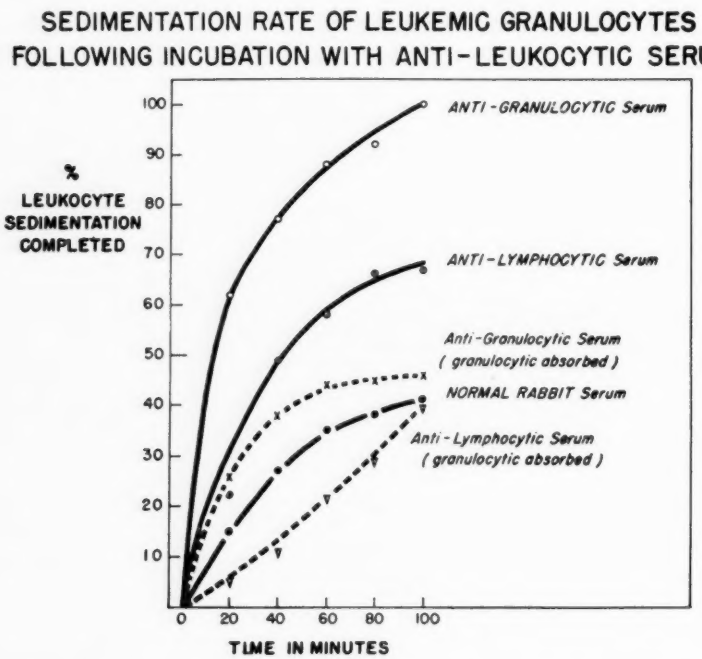

Fig. 13.—After mixing these rabbit sera with plasma suspensions of human granulocytic leukemic cells in a Wintrobe hematocrit tube, the rate of cell sedimentation was observed. At the end of one hour no further leukocyte sedimentation occurred with antigranulocytic serum, and this represented the maximum leukocyte sedimentation. The values obtained for the figure above represent the percentage of sedimentation completed at any specific time with each serum as related to the maximum height of the column of leukocytes sedimented with antigranulocytic serum.

sodium citrate or heparin were employed as the anticoagulant, but not with the balanced oxalate mixture of Heller and Paul. The earliest objective evidence of leukocyte phagocytosis occurred eight to ten minutes after the antileukocytic serum was mixed with the human leukocytes.

DISCUSSION

These studies indicate that a heteroimmune granulocytic leukocyte antibody was produced in rabbits against human leukemic leukocytes. No anti-human lymphocytic antibody was obtained, but the lymphocytic antiserum did contain a small amount of granulocytic leukocyte antibody. These impressions are substantiated by the fact that activity of the antileukocytic sera was not removed through erythrocyte or lymphocyte absorption, but was removed by

human granulocytic leukocyte absorption; activity was not destroyed by heating to 56° C. for thirty minutes; complement was neecssary for leukophagocytosis; and there was a considerable difference in the specificity of action between the two antisera. Furthermore, others[15-39] have prepared experimental antileukocyte sera with similar techniques and have demonstrated specific in vivo and in vitro antileukocytic properties of their antisera. These properties have consisted in vivo of tissue necrosis,[21, 22, 28, 36] leukopenia,[17, 19, 22, 28, 31, 32, 33, 35, 36] and changes in monocytes and granulocytes.[15, 16, 17, 20] In vitro, these sera have produced leukoagglutination,[15, 16, 18, 24, 34] inhibition of leukocyte tissue culture

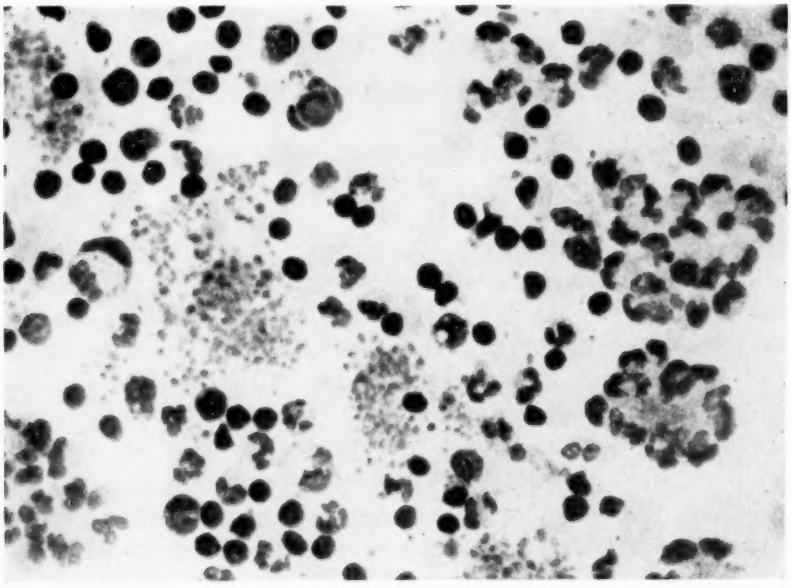

Fig. 14.—Typical rosette and L. E. cell along with the phagocytosis of a polymorphonuclear leukocyte appearing after incubation of L. E. serum with a normal plasma-leukocyte suspension. (×1,200, reduced ⅓.)

growth,[30] complement fixation,[24, 29, 33, 37, 38] destruction of leukocytes,[18, 23, 24, 28, 30, 37, 38] and impairment of phagocytosis of particulate material.[35] In vitro leukophagocytosis has not been demonstrated previously.

The possibility exists that a serum factor with antileukocytic properties was produced in these rabbits in response to the nonspecific stimulus of Freund's adjuvant. However, no antileukocytic action of Coombs' sera prepared by the adjuvant technique employed in these studies could be demonstrated following incubation with human plasma-leukocyte suspensions in comparable concentrations. It has been suggested that heterophil antibody in rabbit serum is responsible for the activity of antileukocytic serum.[26, 27] Although Forssman antibody does exist in normal rabbit serum in variable low titers, these studies and those of others[29] indicate that it alone cannot account for the activity of rabbit antileukocytic sera.

TABLE I. AGGLUTINATION TITERS OF ANTILEUKOCYTIC SERUM WITH LEUKEMIC LEUKOCYTES

ANTIBODY	ABSORBED WITH	ANTIGEN*	MAXIMUM AGGLUTINATION TITER
Antigranulocytic serum	—	Granulocytes	1:1000
Antigranulocytic serum	—	Lymphocytes	0
Antigranulocytic serum	Granulocytes	Granulocytes	0
Antigranulocytic serum	Lymphocytes	Granulocytes	1:1000
Antilymphocytic serum	—	Granulocytes	1:10-1:100
Antilymphocytic serum	—	Lymphocytes	0
Antilymphocytic serum	Granulocytes	Granulocytes	0
Antilymphocytic serum	Lymphocytes	Granulocytes	1:2-1:10
Normal rabbit serum	—	Granulocytes	1:2-1:10

The above figures represent average maximum agglutination titers of three sera obtained from rabbits immunized against either human leukemic lymphocytes or granulocytes.
*Concentration of 50,000 cells per cu. mm.

There are several mechanisms by which leukophagocytosis and agglutination may have occurred. The most likely is that the rabbit serum factor is a specific antihuman leukocyte antibody which rapidly coats human leukocytes during in vitro incubation in the presence of complement. This surface coating with foreign protein alters the cell membrane, and the cells are phagocyted and clumped in response to the foreign surface protein. This reaction is comparable to the mechanism postulated for erythrophagocytosis which occurs in disorders in which serum antibodies are present such as acquired hemolytic anemia[40-42] mismatched transfusion reactions,[43-45] erythroblastosis fetalis,[47-50] and paroxysmal cold hemoglobinuria.[51-54] It is highly probable that the anticomplementary action of the balanced oxalate mixture in the concentrations employed was sufficient to impair leukophagocytosis. Antibody coating of pneumococci and other bacteria in the presence of complement and opsonin are essential before any significant amount of phagocytosis takes place.[55]

It is possible that leukophagocytosis occurs in response to damaged cell membranes from a leukocidin or antibody rather than from surface coating with foreign protein. Alterations in erythrocyte surface structure may result in erythrophagocytosis in paroxysmal nocturnal hemoglobinuria,[56, 57] and following exposure to potassium chlorate,[58, 59] mushroom poisons and male fern extracts,[60] bacterial toxins,[61] and trypsin.[62] However, it is improbable that a leukocidin exists, since it has not been possible to demonstrate complete dissolution of leukocytes upon incubating human leukocytes with these sera or by any of the methods described. Finally, it is possible that this rabbit serum factor acts to stimulate phagocytosis directly, but this would not explain why erythrocytes, platelets, and lymphocytes are spared.

There is much similarity between human L. E. factor and this heteroimmune leukocyte antibody. Both are plasma factors which, in vitro, produce leukocyte clumping, leukocyte destruction, and phagocytosis of leukocytes. Both appear to alter leukocytes from normal subjects, and have similar physical characteristics. Leukocyte antibody produces in vivo leukophagocytosis and leukopenia, and it is very probable that the L. E. factor has a similar effect. On the other hand, certainly they demonstrate striking differences in action. Monocytes and, rarely, neutrophilic granulocytes phagocytize when the anti-

leukocytic serum is used, while the reverse of this is observed with L. E. serum.[63-66] Granulocytes are ingested intact and then destroyed with the antileukocytic serum, whereas with L. E. serum nucleolysis of leukocytes is thought to precede phagocytosis of the nuclear debris.[65, 66] The leukocyte clumping with antileukocytic serum appears to be a simple agglutination phenomenon, while the rosettes of the L. E. test may represent a polymorphonuclear cell foreign body reaction to clumps of nuclear debris.[65, 66] It appears that the L. E. factor is primarily antinuclear in action,[65, 66] whereas the leukocyte antiserum appears to exert more lytic effects on the cytoplasm. In spite of these differences in action, there is enough similarity to suggest that the L. E. factor is, or is closely related to, an autoimmune human leukocyte antibody.

The action of this experimentally produced heteroimmune leukocyte antibody clearly illustrates three mechanisms by which leukocytes may be altered and rendered susceptible to removal from circulation in the human body. The importance of leukophagocytosis per se in the leukopenic mechanism is obscure, but the clumping and damaging of leukocytes would lead to their rapid removal from circulation. It appears probable that these mechanisms may be operating in human disease states as a result of autoimmune antibodies against leukocytes, in a fashion similar to those recognized as operating against erythrocytes and platelets.[67, 68]

SUMMARY

Heteroimmune antibody has been produced in rabbits against human granulocytic leukemic leukocytes. This heteroimmune leukocyte antibody produces in vitro agglutination, lysis, and phagocytosis of normal human granulocytes by monocytes and polymorphonuclear leukocytes. Many of the cells produced upon incubation with antileukocytic serum appeared identical to the "L. E. cell" of Hargraves, and suggests the possibility that the "L. E." factor may be closely related to leukocyte antibody. The action of leukocyte antibody clearly demonstrates three mechanisms by which human leukocytes may be removed from circulation should leukocyte autoimmunization occur.

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OBSERVATIONS ON ISOSENSITIZATION TO THE KELL FACTOR

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IN 1946 Coombs, Mourant, and Race[1] performed the direct antiglobulin test on red cells of newborn babies, and in the course of this investigation encountered a baby whose red cells gave positive reactions even though the mother was Rh positive. The baby was three weeks old and anemic, and was being treated for an enteritis. Further tests by the indirect antiglobulin method showed that the mother's serum contained an antibody which strongly sensitized the father's cells, but did not sensitize the cells of 13 other individuals selected at random. In their conclusions, these authors state: "An antigen and its homologous antibody of a type previously unknown have been disclosed by this technique."

At about the same time, Wiener and Gordon[2] studied an Rh-positive patient who had had a severe intragroup hemolytic transfusion reaction, and detected in this individual's serum an isoantibody which gave reactions unrelated to any previously described blood factor. The antibody failed to clump saline suspended red cells containing the corresponding agglutinogen, except when undiluted serum was used; but by the plasma conglutination method strong clumping was observed, the titer by this method being 24 units. The antibody was designated anti-Si and the corresponding blood factor Si after the patient (Singer) whose serum contained the antibody. Tests on a series of 148 Caucasoids showed the frequency of this blood factor to be about 12.9 per cent. Moreover, studies on 12 families with 25 children showed the agglutinogen Si to be inherited as a simple Mendelian dominant.

In the meantime, the British investigators[3] continued their study of the agglutinogen which they had discovered and named the Kell agglutinogen, and they found at first its incidence to be about 7 per cent among random bloods. A sample of anti-Si serum was sent to Dr. A. E. Mourant in London, who tested it in parallel with anti-Kell serum and found the two antibodies to be identical.[4] Moreover, tests on 423 individuals showed the incidence of the Kell agglutinogen to be 10 per cent,[5] or about the average of the frequencies originally reported by the independent discoverers in England and America. Accordingly, Wiener has adopted the name Kell for the new agglutinogen in place of Si, and by mutual consent this name has been shortened to the symbol K. In this paper the blood factor (and agglutinogen) and its corresponding antibody will therefore be designated as K and anti-K, respectively. More recently, in a study on 56 families with 119 children, Sanger and associates[6] have corroborated the previous report of Wiener and Gordon that the agglutinogen K is inherited as a simple Mendelian dominant.

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The announcement of the discovery of the Kell (or Si) agglutinogen was soon followed by reports by other investigators of additional instances of erythroblastosis and transfusion reactions caused by sensitization to the agglutinogen K. In fact, within less than two years, as many as 16 examples of the antibody were recognized, and these reports have been summarized by Race and Sanger.[5] In several of these cases the patient's serum contained in addition to anti-K, antibodies for the Rh_0 factor. More recently Simmons and Jakobowicz[7] encountered three patients within a short period of time, each of whose sera contained anti-K together with anti-Rh_0 and anti-rh′.

According to Race and Sanger,[6] blood factor K is almost as antigenic as blood factor Rh_0. The lower incidence of clinical examples of K sensitization is due, at least in part, to the fewer opportunities for sensitization to develop, in view of the relatively low incidence of type K bloods.*

In addition to the reports already cited above, Wiener and co-workers[8] have recently encountered another example of Kell sensitization causing hemolytic transfusion reactions in a patient with thrombotic thrombocytopenic purpura and hemolytic anemia. In the present paper we propose to describe two additional examples of Kell sensitization; in one case giving rise to a hemolytic transfusion reaction, the other being detected in a case of erythroblastosis. The case of erythroblastosis is of special interest, since the mother's serum contained in addition to anti-K, the rare antibody anti-rh^w.

CASE REPORTS

CASE 1.—(Bay Ridge Hospital, service of Dr. Ralph H. Gralick.) Our attention was first called to this patient, a woman 35 years of age, on Dec. 30, 1952, one day after she had had a hemolytic transfusion reaction. When the transfusion was started the day before, the patient complained of back pain and the transfusion was stopped. After a short time the infusion was started again and 200 ml. of blood were administered, after which the transfusion was discontinued because of the occurrence of a severe chill. The next morning the patient was observed to be jaundiced, and then one of us (G. J. B.) was requested to investigate the problem.

The patient had the following obstetrical history. Her first pregnancy was terminated by cesarean operation with the birth of a female hydrocephalic infant, who died. There were three additional pregnancies, all terminated by cesarean section. The second and third pregnancies yielded two normal children, now 5½ and 4 years old, respectively. The fourth pregnancy was also terminated by cesarean operation with the birth of a normal female infant on Dec. 3, 1952, that is, almost a month before the hemolytic transfusion reaction occurred.

The transfusion history was as follows: the first blood transfusion the patient received was after the first cesarean operation. In 1950, the patient fractured her leg and was given two more blood transfusions, both of which were followed by chills. At the time of the most recent cesarean operation on Dec. 3, 1952, the patient bled so profusely that it was necessary to perform a hysterectomy. Within a period of forty-eight hours she was given 9 pints of blood, most of the blood being given while she was under anesthesia. The patient then developed almost complete anuria, passing only about one ounce of urine a day for a number of days. The blood creatinine concentration reached as high as 15 mg. per 100 ml. Thereafter urinary output gradually increased, became profuse by the eleventh day, after which the patient gradually improved. However, she was observed to be pale, and on December 18 she was given another blood transfusion, which was followed by a

*Kell-positive blood is designated as type K; Kell-negative blood as type kk.

reaction. The patient continued to improve except that her pallor increased. Then on December 29 the blood transfusion was given which caused the hemolytic reaction that attracted our attention to the case.

Examination of the patient's blood showed her to belong to group O, but the Rh type was not entirely clear at first, because of the presence of foreign blood cells from the transfusions she had received. By the antiglobulin technique, the patient's serum was found to clump strongly the red cells of the donor whose blood had caused the reaction. Tests for the factor K showed the patient to be kk and the donor type K, and tests carried out in parallel with standard anti-K proved the abnormal antibody in the patient's serum to be anti-K. On the strength of these findings, the patient was given two additional transfusions of group O blood from type kk donors, and these transfusions caused no untoward reaction (see Table I).

TABLE I. RESULTS OF TYPINGS ON PATIENT (CASE 1) AND DONORS CARRIED OUT AFTER HEMOLYTIC TRANSFUSION REACTION

BLOOD OF	GROUP	M-N TYPE	Rh-Hr TYPE	KELL TYPE
Patient	O	Mixture	Rh_1rh (?)	kk
Donor whose blood caused hemolytic reaction	O	N	Rh_1rh	K
Two compatible donors later selected and used without reaction	O	MN	Rh_1Rh_1	kk
	O	MN	Rh_0	kk

Titration proved that the K antibody in the patient's serum had a titer of 500 to 1,000 units (see Table II). Tests were then carried out on the husband and children of the patient in order to determine whether her pregnancies had anything to do with the sensitization to the factor K. It was found (Table III) that all members of the family belonged to type kk, eliminating this possible source of isosensitization.

TABLE II. TITRATION OF KELL ANTIBODY IN SERUM OF PATIENT (CASE 1)

BLOOD CELLS OF	REACTION BY ANTIGLOBULIN METHOD OF SERUM DILUTIONS											
	UNDIL.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Incompatible donor of Table I	++±	++±	++±	++±	++	++	++	++	++	+±	±	–
Known type K	++±	++	++	++	++	++	++	++	+±	+±	+	–
Compatible donors of Table I	–	–	–									
	–	–	–									
Known type kk	–	–	–									

TABLE III. RESULTS OF TYPINGS ON FAMILY OF PATIENT (CASE 1)

BLOOD OF	A-B-O GROUP	M-N TYPE	Rh-Hr TYPE	KELL TYPE
Father	A_1	M	Rh_2	kk
Mother (patient)	O	N	Rh_1rh	kk
1st child, 5½ yr. ♀	O	MN	Rh_2	kk
2nd child, 4 yr. ♀	O	MN	Rh_2	kk
3rd child, newborn ♀	A_1	MN	Rh_0	kk

A puzzling aspect of the case was the result of the direct antiglobulin test on the patient's red cells. The reaction of a sample of the patient's red cells obtained before the blood transfusion was negative, while the direct antiglobulin test on the post-transfusion sample was strongly positive, the degree of coating being 24 per cent, as compared to Rh-positive cells maximally coated with Rh antibodies[9] (see Table IV). Since type K cells maximally coated with K antibody exhibit a degree of coating equal to approximately 25 per cent of maximally sensitized Rh-positive cells (Wiener and Gordon[9]), this suggested that the positive antiglobulin reaction obtained with the patient's cells after the transfusion might be due to donor's cells coated with K antibodies, and surviving in the patient's circulation. However, no unagglutinated red cells were seen in the direct antiglobulin test, though this was to be expected since the patient's own cells were type kk. We therefore considered the possibility that the reaction was due to an autoantibody, but this appears to be excluded by the observation that the direct antiglobulin test was negative on the pretransfusion sample and positive on the post-transfusion sample. Besides, when the direct antiglobulin test was repeated at intervals after the transfusion, it was observed to become gradually weaker and finally completely negative within a space of two months (cf. Table IV). This is more readily explained on the assumption of elimination of donor's coated red cells from the circulation, rather than on the basis of autosensitization, since autosensitization usually tends to persist for a period of months or years. Perhaps the puzzling observations in the differential agglutination tests were due to some technical difficulty incidental to the delicate antiglobulin technique.

TABLE IV. RESULTS OF QUANTITATIVE DIRECT ANTIGLOBULIN ON PATIENT'S (CASE 1) CELLS, BEFORE AND AFTER TRANSFUSION OF INCOMPATIBLE TYPE K CELLS

TIME OF TEST	TITER (UNITS) OF ANTIGLOBULIN SERUM FOR		DEGREE OF COATING* (PER CENT)	ICTERUS INDEX (UNITS)
	PATIENT'S CELLS	MAXIMALLY COATED RH-POSITIVE CELLS		
Dec. 30 (pretransfusion)	0	46	0	—
Dec. 30 (posttransfusion)	11	46	24	20
Jan. 3	22	46	48	8
Jan. 14	6	46	13	4
Jan. 27	4	36	11	—
Mar. 5	0	50	0	—

*The degree of coating is calculated by dividing the titer of an antiglobulin serum for the patient's washed red cells by its titer for maximally coated washed Rh-positive cells, and then multiplying by 100.

It might be pointed out that if the patient had not had the hysterectomy, the K sensitization would not have interfered with subsequent pregnancies, since all the children in the family would have to belong to type kk. As in the case of Rh sensitization,[10] a type kk newborn baby of a sensitized type kk mother would be expected to have K antibodies in his serum with a titer equal to that of the mother's serum. This suggested that it might be determined when the high-titered K antibodies first appeared in the mother's serum by testing the blood of her newborn baby. Tests on the baby showed the presence of K

antibodies, but in a very low titer only, namely, 1 to 2 units. Assuming the half-life of K antibodies, like that of Rh_0 antibodies,[11] to be approximately one month, and considering that the baby was a month old when tested, the titer of the K antibodies in the maternal serum at term probably did not exceed 4 units.* Therefore, one of the numerous transfusions she received at the time of the cesarean operation and hysterectomy must have been from a type K donor and stimulated the rise in titer of the K antibodies in her serum. Unfortunately, it was not possible to trace the donors used for the earlier transfusions in order to type them for the K factor.

Since uncontrollable bleeding and anuria may be sequelae of transfusions of incompatible blood, one might suspect that the patient had an unrecognized transfusion reaction at the time of operation. This does not seem likely, however, considering the low titer (only 4 to 8 units) her K antibodies must have had at that time. A more likely explanation of the continuous bleeding and anuria is the entrance of thromboplastic material into the maternal circulation, resulting in toxemia and afibrinogenemia.[12]

The last time the patient was observed was four months after the blood transfusion. She reported that since leaving the hospital she has been well and able to carry out her household duties. A blood count showed only a mild anemia. Periodic antibody titrations up to three months after the transfusion showed little, if any, drop in the titer of the K antibody in her serum. The patient has been warned that if she requires any further transfusions, she is to receive only type kk, group O blood.

CASE 2.—(Jewish Hospital of Brooklyn, service of Dr. Richard Waltman.) This patient was a newborn male infant, who developed jaundice shortly after birth. A blood count showed a hemoglobin concentration of 18 Gm. per cent, with 16 nucleated red cells per 100 W.B.C. on the smear. A routine direct antiglobulin test was reported to be positive. Since the mother was known to be Rh positive, the problem was then referred to one of us (A.S.W.) for more complete studies.

With regard to the mother's history, she had never received a blood transfusion and had had only one previous pregnancy. This terminated with the birth of a full-term normal female infant, who was 6 years old and well. The second pregnancy resulted in the birth of the patient. This pregnancy had been uneventful and, since the mother had been found to be Rh positive, no antibody tests had been done on her serum. The patient was four days old when first seen by us.

The blood of the mother proved to belong to group A_1 M Rh_1Rh_1, the baby's blood to group O M Rh_1Rh_1. This confirmed the previous report that the mother was Rh positive, and appeared to exclude the possibility of Rh-Hr sensitization. The direct antiglobulin test on the baby's red cells was found to be strongly positive, the degree of coating being approximately 33 per cent, compared to cells maximally coated with Rh_0 antibody. As has already been pointed out, cells maximally coated with K antibody have about this degree of coating, so that this suggested the possible presence of K sensitization. Indeed, it was found that the mother belonged to type kk and the father and first child to type K. The blood of the patient himself could not be typed for the K factor, because his cells were already coated with antibody, and the only anti-K serum

*If, as seems to have been true in Case 2, the half-life of the K antibodies here was only fifteen days, this would make the maternal titer at term equal to 8 units.

available could be used only by the conglutination or antiglobulin technique. Moreover, direct matching of the mother's serum with the father's blood cells by the antiglobulin technique showed the presence of an abnormal isoantibody of high titer, and a similar antibody was detected in the serum of the baby. On the basis of these findings we concluded that the baby had mild erythroblastosis caused by K sensitization, and it was predicted that he would eventually develop a hemolytic anemia.

The jaundice subsided within a few days. The baby remained apparently well and was discharged from the hospital with his mother to be followed up in the office. The red cell count and hemoglobin concentration dropped gradually in accordance with expectation, so that by the time the baby was two weeks old, the hemoglobin concentration was only 56 per cent (8.1 Gm.) with 3 million R.B.C. per cubic millimeter. A transfusion of 65 ml. of packed red cells from group O MN Rh_1rh kk donor was then given, after which the hemoglobin concentration rose to 11.1 Gm. (77 per cent) with 4.1 million R.B.C. per cubic millimeter. Again the blood count gradually fell, so that by the time the baby was a month old, the hemoglobin concentration was again 56 per cent (8.1 Gm.) with 3 million R.B.C. per cubic millimeter. A second tranfusion of 75 ml. of packed red cells from a group O M Rh_1Rh_2 kk donor was then given, and no further treatment was required.

During this period of observation comparative titrations were carried out on the sera of the mother and baby against the husband's cells (see Table V). Considering that the titration method in general has a technical error of at least one serum dilution (or 100 per cent), the results shown in Table V indicate that the titer of the antibodies in the mother's serum showed no significant change for three months following the patient's birth, the average titer being 500 units. The titer of the baby's serum, on the other hand, fell from its initial level of 250 units to a titer of only 2 units by the time the baby was three months old. If K antibodies had a half-life of approximately thirty days like Rh_0 antibodies,[11] one could expect the titer to have been 30 units instead of 2 units at the time of the last examination. As will be shown later, the half-life of K antibodies, at least in the present case, appears to have been fifteen days.

TABLE V. PERSISTENCE OF ABNORMAL ISOANTIBODIES IN SERA OF MOTHER AND BABY (CASE 2)

DATE OF TEST	BABY'S AGE (DAYS)	TITER BY ANTI-GLOBULIN METHOD FOR FATHER'S CELLS OF SERUM OF	
		MOTHER	BABY
Dec. 1	4	960	250
Jan. 8	42	250	60
Jan. 23	57	480	15
Feb. 20	85	480	2

The mother's serum was tested in parallel with a standard anti-K serum against a large series of blood specimens. The two sera gave similar reactions except for occasional blood specimens which reacted with the mother's serum but not with the standard anti-K serum. This suggested that the mother's serum contained a second antibody besides anti-K, and the low percentage of the discrepancies indicated that the second blood factor had an even lower frequency

than K. Since the blood factor rh^w has a low frequency of about the correct order, the bloods of the patient's family were tested for this factor. As anticipated, the mother proved to the rh^w negative, while the father and both children were rh^w positive (see Table VI). Further tests carried out in parallel with anti-rh^w serum confirmed the assumption that the mother's serum contained the anti-rh^w antibody as well as anti-K. It should be pointed out that the rh^w antibody in the maternal serum produced clumping by the saline agglutination method as well as the antiglobulin technique, while the K antibody produced clumping only by the anti-globulin method as shown in Table VII. Unfortunately, by the time that this was discovered the baby was three months old, so that it was no longer possible to test his serum for the K and rh^w antibodies separately.

TABLE VI. RESULTS OF TYPINGS ON ERYTHROBLASTOTIC BABY'S (CASE 2) FAMILY

BLOOD OF	A-B-O GROUP	M-N TYPE	Rh-Hr TYPE	KELL TYPE
Father	O	M	$Rh_1^wRh_1$	K
Mother	A_1	M	Rh_1Rh_1	kk
1st child, 6 yr. ♀	O	M	$Rh_1^wRh_1$	K
2nd child, newborn ♂ (patient)	O	M	$Rh_1^wRh_1$	?

TABLE VII. TITRATION OF THE rh^w AND KELL ANTIBODIES IN SERUM OF MOTHER OF ERYTHROBLASTOTIC BABY (CASE 2)

METHOD OF TITRATION	BLOOD CELLS OF	REACTION WITH SERUM DILUTED								
		UNDIL.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Saline agglutination	Father ($Rh_1^wRh_1$ K)	++	++	++	++	+±	tr.	−		
	M. S. (Rh_1rh K)	−	−	−						
	I. B. (Rh_1^wrh kk)	++	++	++	++	+±	tr.	−		
	Known rh^w-neg. and type kk	−	−	−						
Antiglobulin	Father ($Rh_1^wRh_1$ K)	++±	++±	++±	++±	++	++	+±	tr.	−
	M. S. (Rh_1rh K)	++±	++±	++±	++	++	++	+±	tr.	−
	I. B. (Rh_1^wrh kk)	++±	++±	++	++	++	++	+±	tr.	−
	Known rh^w-neg. and type kk	−	−	−						

As has already been mentioned, it was impossible at first to test the baby's red cells for the K factor, because the cells were coated with antibodies, and the only anti-K reagents available to us had to be used by the antiglobulin method or the conglutination method. On the other hand, there was no difficulty in testing the baby's cells for the rh^w factor, since an agglutinating anti-rh^w serum was available (see Table VI).* For several months after birth the presence of transfused red cells interfered with the test for the K factor. When finally these had been replaced by the baby's own red cells, and the direct

*For this serum we are indebted to Dr. J. J. van Loghem, Jr.

antiglobulin test was no longer positive, we were surprised to find that the baby actually belonged to type kk. Thus, the Kell sensitization of the mother was not the cause of the baby's illness, but instead the originally unsuspected rh^w sensitization.

Since the baby belongs to type kk, one would have expected the titer of the K antibodies in the serum of the baby at birth to equal the titer of the mother's serum. Actually, we found the titer of the mother's serum to be 960 units, while the titer of the baby's serum was only 250 units (Table V). In evaluating these results one must allow for limitations in the technique, as well as for the fact that the father's red cells were used for the titrations. Thus, the titer of the mother's serum really represented the combined effect of the rh^w and K antibodies, while this was not true of the baby's serum, since at least part of the rh^w antibodies had been absorbed onto the baby's red cells.

With regard to the relatively rapid drop of the K antibodies in the baby's serum, one might conceive that the baby's rh^w-positive cells were absorbing the rh^w antibodies in his serum and that the K antibodies were absorbed at the same time, assuming that they were "linked" to the rh^w antibody. However, for three months after birth the baby's marrow released hardly any new red cells and most of the cells in the baby's circulation were the rh^w-negative and K-negative cells received by transfusion. Moreover, absorption experiments on the mother's serum failed to show any evidence of linkage between the rh^w and K antibodies it contained. The correct explanation appears to be, as already mentioned, that the K antibodies in the baby's serum had a half-life of only approximately fifteen days, instead of the expected half-life of thirty to thirty-five days, based on studies on Rh antibodies.

The Blood Factor k (Cellano).—Levine[13] has demonstrated the existence of an agglutinogen k (or Cellano) related to agglutinogen K, serologically and genetically, in the same way that the agglutinogens M and N are related to one another. By testing with both antisera, anti-K and anti-k, three types can be distinguished, namely, KK, Kk, and kk. From the frequency of agglutinogen K, one can calculate the frequencies of the genes K and k, and from this in turn predict the frequency of type KK.

For example, taking the frequency of type K to be approximately 10 per cent, and type kk 90 per cent,

Then, $k = \sqrt{kk} = \sqrt{0.90} = 0.95$, approximately

Therefore, $K = .05$

Accordingly, type KK $= (K)^2 = (.05)^2 = 0.0025$, or 0.25 per cent

It was therefore predicted that anti-K serum would fail to react with only one of about 400 blood specimens. Indeed, Levine found that his anti-K serum agglutinated 99.8 per cent of the blood specimens he tested.

Because of the low frequency of type KK blood, families illustrating the segregation of genes K and k, in which this type occurs, are quite rare. Levine[14] has collected five examples of the mating Kk × Kk and among 23 children found 11 type KK. The excess of type KK children above the 25 per cent expected Levine attributed to his selection of families through children of type KK. In view of the rarity of such families, it may be of interest to add another such family encountered by one of us (W). As shown in Table VIII, in this Kk

× Kk family there were two children of type kk and one type KK. (The anti-K serum used for these tests was kindly provided by Dr. P. Levine.) Additional families with the rare type KK have recently been reported by Shapiro.[14a]

TABLE VIII. FAMILY ILLUSTRATING HEREDITARY TRANSMISSION OF K-k TYPES

BLOOD OF	A-B-O GROUP	M-N TYPE	Rh-Hr TYPE	P TYPE	DUFFY TYPE	KELL TYPE
Father	O	M	Rh_1Rh_1	P	F	Kk
Mother	A_1	MN	rh″	P	F	Kk
1st child	A_1	M	Rh_1rh	pp	F	kk
2nd child	O	MN	Rh_1Rh_2	P	F	kk
3rd child	O	MN	Rh_1Rh_2	P	F	KK

COMMENT

In isoimmunization studies on the Rh_0 factor[15-17] it was found that in general at least three contacts with the antigen are required before hemolytic transfusion reactions are likely to result. The first contact with the Rh_0 antigen, either as a result of an injection of blood or a pregnancy, "primes" the patient, so that after a suitable interval (at least four months), a second exposure to the antigen may stimulate the appearance in the serum of the Rh_0 antibody, and, then, finally, a transfusion of Rh-positive blood at the time when the antibody titer is at its peak may give rise to a hemolytic transfusion reaction. Assuming that isoimmunization to the blood factor K can be produced by a similar process, the chance of a hemolytic transfusion reaction would depend on the frequency of the Kell factor in the population and its antigenicity, a_K. Thus, the chance of a transfusion reaction resulting from sensitization to blood factor K, T_K, can be expressed in the terms of a_K as follows, taking the frequency of the K factor in the population to be 10 per cent:

$$T_K = \frac{9}{10} \times \frac{1}{10} \times \frac{1}{10} \times \frac{1}{10} a_K = 0.0009 a_K$$

Similarly, in the case of the Rh_0 factor, taking the frequency of the Rh_0 factor to be $\frac{1}{7}$,

$$T_{Rh} = \frac{1}{7} \times \frac{6}{7} \times \frac{6}{7} \times \frac{6}{7} a_{Rh} = 0.09 a_{Rh}$$

Therefore, assuming that the antigenicity of the blood factor K is equal to the antigenicity of the Rh_0 factor, these calculations indicate that there would be only one hemolytic transfusion reaction caused by isosensitization to the factor K for every 100 caused by Rh_0 sensitization, if the K and Rh_0 factors were disregarded when selecting donors for blood transfusion. The calculations which have been made regarding hemolytic reactions apply to males and nonpregnant women. In cases where sensitization by pregnancy underlies the occurrence of a hemolytic transfusion reaction in women, one must allow for the fact that almost all type K husbands are heterozygous, while about two-fifths of Rh-positive husbands are homozygous. This would tend to increase still further the disparity in the incidence of transfusion reactions.

With regard to erythroblastosis fetalis, considering the common case where the second child is affected and the mother has received no injection of blood

which might have sensitized her, this generally occurs only if the first two children both possess the blood factor lacking from the mother's blood. The incidence of such families in the case of Rh sensitization is:[18]

$$\frac{1}{7} \times \frac{6}{7} \times \left(\frac{2}{5} + \frac{1}{4} \times \frac{3}{5} \right) = \frac{33}{490} = 0.067$$

In the case of the factor K, the incidence of families of this type is:

$$\frac{9}{10} \times \frac{1}{10} \times \frac{1}{4} = \frac{9}{400} = 0.022$$

On this basis, assuming equal antigenicity for the K and Rh_0 factors, we would expect one-third as many cases of erythroblastosis caused by K sensitization as by Rh_0 sensitization. That in clinical practice instances of K sensitization appear to be much rarer may be due, in part, to the fact that erythroblastosis caused by K sensitization would be likely to be milder, because of the smaller number of antigenic points on the red-cell envelope,[9] and such cases therefore might be overlooked. In some such cases in the past, the slowly developing anemia may have been overlooked until it was too late, and the death incorrectly attributed to respiratory infection because of the baby's dyspnea which was actually caused by extreme anemia. In addition, the assumption that K is as good an antigen as Rh_0 is probably incorrect.

SUMMARY

A hemolytic transfusion reaction caused by sensitization to the blood factor K is described. Pregnancy was excluded as playing any role in isosensitizing the patient, since the patient's children all belonged to type kk. The patient had, however, received three transfusions in previous years, and nine additional transfusions at the time of a cesarean operation one month before the blood transfusion which caused the hemolytic reaction. By the antiglobulin technique anti-K antibodies of a titer of 500 to 1,000 units were demonstrated in this patient's serum. Two transfusions of type kk blood were then given without any untoward reaction, and the patient made an uneventful recovery.

The blood cells of a jaundiced Rh-positive baby of an Rh-positive mother gave a positive direct antiglobulin reaction, which was explained by the demonstration of high-titered K and rh^w antibodies in the serum of the mother. The hemolytic anemia which resulted in the baby responded to two simple transfusions of type kk, rh^w-negative blood. In this case, the isosensitization was traced to the single preceding pregnancy, since the mother had never received a blood transfusion. Later it was shown that the baby though rh^w positive belonged to type kk, so that its illness was due entirely to the rh^w antibodies and not to the K antibodies. Absorption tests show no evidence of linkage between the rh^w and K antibodies in the mother's serum. The curve of elimination of the anti-K antibodies passively acquired by the type kk baby from the mother showed a half-life of approximately fifteen days, or about half the value previously obtained for Rh_0 antibodies. In the mother, on the other hand, the antibody titer remained relatively constant at a titer of about 500 units, during a period of observation of about three months.

A Kk × Kk family is described which illustrates the Mendelian segregation of the blood factors K and k, with the appearance of the rare type KK in one child.

It is calculated that assuming the blood factor K to be equal in antigenicity to the Rh_0 factor, if the K and Rh_0 factors were disregarded when selecting donors for blood transfusion, there would be only one hemolytic transfusion reaction caused by K isosensitization for every 100 hemolytic transfusion reactions caused by Rh_0 sensitization, in view of the difference in distribution of these blood factors in the population.

It is also calculated that the incidence of erythroblastosis caused by K sensitization should be about one-third as high as cases caused by Rh_0 sensitization. That this has not been the case in clinical practice may be due to the fact that such cases tend to be mild, and to manifest themselves as a late hemolytic anemia. Thus, the patient may be discharged without this condition being recognized, and the illness which later develops may be attributed to some other mechanism. Or else, one must conclude that K is less antigenic than Rh_0.

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THE EFFECT OF HOMOLOGOUS BONE MARROW EMULSION ON RABBITS AFTER TOTAL BODY IRRADIATION

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WITHIN the past few years a number of fundamental contributions have been made in regard to the problem of increasing the survival of animals receiving lethal amounts of total body irradiation. Congdon[1] and Jacobson[2] have exhaustively reviewed these reports in recent publications. In general, increased survival has been attained by the use of a wide variety of substances and techniques employed before, during, or after exposure. However, the reason for the increased survival rate is still not clearly defined, as indicated by the various hypotheses advanced.

This present report deals principally with the effect on survival of bone marrow emulsion and related substances administered intravenously after total body irradiation to rabbits. The more fundamental problems concerning the response of the entire hematopoietic system will be considered in detail at a later date.

METHODS

For this study 180 New Zealand white rabbits were used. Male and female animals were divided about equally in the various experimental groups. At the time of irradiation their age was ten to twelve weeks, and the average weight was 2.5 kilograms. They were housed separately in wire cages and fed Purina rabbit chow and water in individual dishes ad libitum. Weight was recorded daily. Total white blood counts and differentials were carried out each day and red blood counts were determined weekly. Complete autopsies were carried out on all animals dying.

Each animal received 1,200 r measured in air of single total body irradiation, 600 r to each side of the body. Factors were as follows: 200 kilovolts, 15 milliamperes, with added filtration of 0.5 mm. of Cu and 1.0 mm. of Al, giving a half-value layer of 1.2 mm. of Cu. The dose rate was 20 r per minute at 50 cm. distance, exposure being measured with a Victoreen ionization chamber.

Intravenous injections of homologous serum, homologous bone marrow emulsion in serum, or the supernate of such an emulsion were given.

The marrow emulsion was prepared in the following manner. Another normal adult rabbit of the same breed and age was bled rapidly from the marginal ear vein, the blood collected in a sterile tube, centrifuged at 3,000 r.p.m. for fifteen minutes, and the clear serum drawn off. This animal was then killed by air embolism, the marrow from both femurs removed, and emulsified gently in the animal's own serum. After gentle shaking by hand to mix it well, it was immediately injected into the marginal ear vein of the previously irradiated recipient animal. Because of the absence of bony spicules in the

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rabbit's femur this procedure was accomplished easily and quickly, an average of ten minutes intervening between the sacrifice of the donor rabbit and injection of the emulsion into the recipient. Just before injection, a count of the emulsion was done to determine the number of nucleated cells per cubic centimeter so that the total number of cells injected could be determined. Differential counts were carried out on smears of the emulsion. The relative proportion of the various nucleated cells present in the intact marrow, as determined by direct marrow smears, was observed in the emulsion. We have found that the use of serum, rather than saline or some other electrolyte solution, provides for the best preservation of individual cells. The majority of cells in the emulsion are single, rather than in clumps, and very few are broken up or disintegrated. Sterile precautions were observed throughout the procedure, and the serum and emulsion were kept at as near body temperature as possible. Over one hundred such injections have been carried out rapidly without incident except for one probable case of cerebral embolism; bony spicules have rarely been observed in the lungs of recipient animals.

The supernate of the marrow emulsion was prepared by centrifuging the emulsion immediately after preparation for fifteen minutes at 3,000 r.p.m. and then drawing off the clear supernate. Counts on the supernatant fluid before injection into the recipient animals rarely revealed any cells. Injection into the irradiated animal was through the marginal ear vein, and no untoward reactions were observed.

Animals were divided into six groups for this study as indicated in Tables I and II. Group I was subdivided into three parts in order to compare the effect of marrow given at various times after irradiation with a comparable control group. Thus, Group III, treated within an hour of exposure, can be compared with the entire control group of 60 animals. Group IV, treated one day after exposure, is compared with the 28 animals of the control group surviving at least one day, and Groups II, V, and VI, treated three days after irradiation, are compared with the 21 animals living at least three days. This breakdown is necessary, since to evaluate the effect of treatment at any given interval after exposure, only those control animals living at least an equal interval from irradiation to the time of treatment can be considered for comparison.

RESULTS

Control Group Receiving 1,200 r of Total Body Irradiation Only (Group I).—The survival figures for this group are given in Table I. Under the

TABLE I. SURVIVAL OF CONTROL ANIMALS (GROUP I) AFTER 1,200 R TOTAL BODY IRRADIATION. GROUP DIVIDED ACCORDING TO MINIMAL SURVIVAL TIME AFTER IRRADIATION

NUMBER OF ANIMALS	MINIMAL SURVIVAL TIME	PER CENT 30-DAY SURVIVAL
60	1 hour	8.3
30	1 day	16.7
24	3 days	20.8

TABLE II. EFFECT ON SURVIVAL OF HOMOLOGOUS BONE MARROW EMULSION AND RELATED SUBSTANCES ADMINISTERED INTRAVENOUSLY AFTER 1,200 R TOTAL BODY IRRADIATION

GROUP	NUMBER OF ANIMALS	MATERIAL	TIME OF INJECTION	PER CENT 30-DAY SURVIVAL
II	10	Serum	3 days after irradiation	30.0
III	20	Marrow Emulsion	Within 1 hour after irradiation	20.0
IV	30	Marrow Emulsion	1 day after irradiation	26.6
V	30	Marrow Emulsion	3 days after irradiation	60.0
VI	30	Supernate of marrow emulsion	3 days after irradiation	53.3

conditions of this experiment, 91.7 per cent of all control rabbits irradiated died before thirty days. One-half of these animals died within a twelve-hour period after irradiation; the remaining animals died at various times during the two-week period after exposure, the greatest number of deaths occurring eleven and twelve days after irradiation. At death all animals were autopsied, and in most instances we were unable to demonstrate any organic cause for death. Postmortem blood cultures were negative, and, as is to be expected in the rabbit, hemorrhagic tendencies of more than a minor degree were not seen. In five of the entire series of 180 animals ulcerations of the gastrointestinal tract, with complicating perforation and peritonitis, were present,

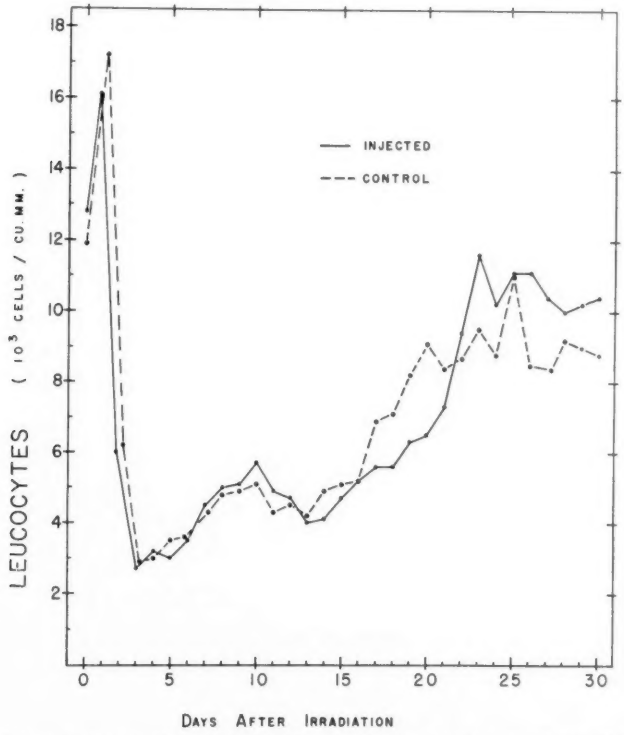

Fig. 1.—Total leukocyte counts of all rabbits receiving marrow emulsion three days after irradiation compared with control group surviving at least three days.

and undoubtedly were the cause of death. This occurred, however, in the third and fourth weeks after exposure, as did several cases of pneumonitis. Although probably related to the irradiation, they were found only rarely, and seem to be an incidental factor in the over-all problem of radiation death in rabbits.

Figs. 1 to 4 illustrate the peripheral blood picture in regard to total leukocytes, neutrophils, lymphocytes, and erythrocytes. A marked neutrophilic leukocytosis in the first twenty-four hours, followed by a sharp drop in the next forty-eight hours, was characteristically seen in the rabbit after this level of total body irradiation. In the succeeding four or five days there was

a gradual rise in the absolute number of neutrophils to approximately preirradiation levels, followed by another drop, with normal levels being regained toward the end of the third week. We have observed that animals die at all points along this curve, and, although more die toward the end of the second week, there is no definite correlation between death and the level of circulating neutrophils. Furthermore, we have rarely seen evidence of infection in those animals dying, even during the leukopenic periods.

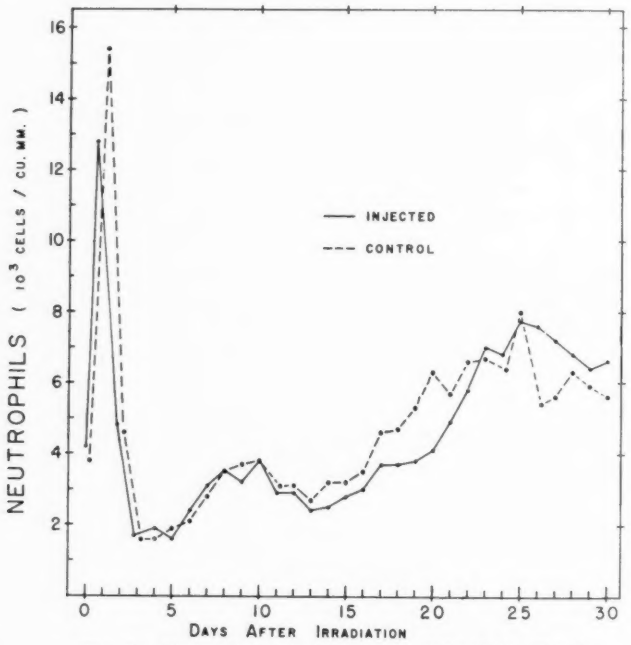

Fig. 2.—Neutrophil counts of same animals in Fig. 1.

The absolute number of circulating lymphocytes dropped precipitously in the first twenty-four hours after irradiation, and then increased slowly during the next thirty days, returning to only 35 per cent of the preirradiation level at the end of one month. Thereafter, in surviving animals the increase was steady, original levels being regained by the end of the second month. The erythrocyte count can be seen to follow a downward curve after the first week, the lowest level being reached five weeks after exposure. Even at the end of two months, normal levels had not been regained.

The most significant difference between animals dying and surviving was found in the weight curves (Fig. 5). Those animals dying lost weight steadily until death, whereas those surviving began to regain weight after two weeks and steadily increased thereafter. Characteristically, those animals eventually dying refused food and water after the first few days.

Rabbits Receiving Homologous Serum Three Days after Irradiation (Group II).—The thirty-day survival for this group was not significantly increased over that of the control animals living at least three days after exposure.

Comparison of the peripheral blood pictures of these two groups revealed no differences, and there was no indication that the serum had in any way altered the postirradiation course. The amount of serum administered, as indicated in Table III, corresponded to the quantity of marrow emulsion given at this time period after irradiation.

Rabbits Receiving Homologous Bone Marrow Emulsion Within One Hour after Irradiation (Group III).—There was no indication that treatment at this time interval altered the expected mortality to any significant degree. Peripheral blood counts in all respects corresponded closely to those of other groups, and there was no evidence in those animals surviving of earlier regeneration of the hematopoietic system.

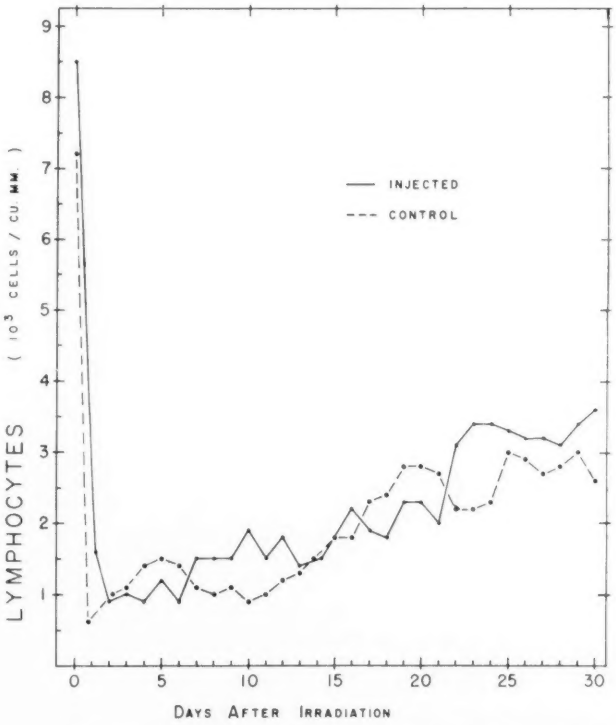

Fig. 3.—Lymphocyte counts of same animals in Fig. 1.

TABLE III. AVERAGE AMOUNT OF FLUID AND NUMBER OF NUCLEATED CELLS INJECTED INTO THOSE ANIMALS SURVIVING THE THIRTY-DAY PERIOD AFTER IRRADIATION AND THOSE ANIMALS DYING DURING THIS PERIOD

GROUP	MATERIAL	AMOUNT IN C.C.		TOTAL NUCLEATED CELLS	
		SURVIVING	DYING	SURVIVING	DYING
III	Emulsion	10.5	11.0	10.40×10^8	9.28×10^8
IV	Emulsion	9.5	8.8	7.78×10^8	7.98×10^8
V	Emulsion	7.9	8.8	6.26×10^8	6.82×10^8
II	Serum	7.4	6.8		
VI	Supernate of emulsion	12.2	11.3		

Rabbits Receiving Homologous Bone Marrow Emulsion One Day after Irradiation (Group IV).—Treatment at this time interval after irradiation increased survival only slightly over the preceding group. When compared with the comparable control group, no significant increase was noted. Again, the peripheral blood count in all respects corresponded to that of the control groups, and no evidence of earlier regeneration of the hematopoietic system was observed.

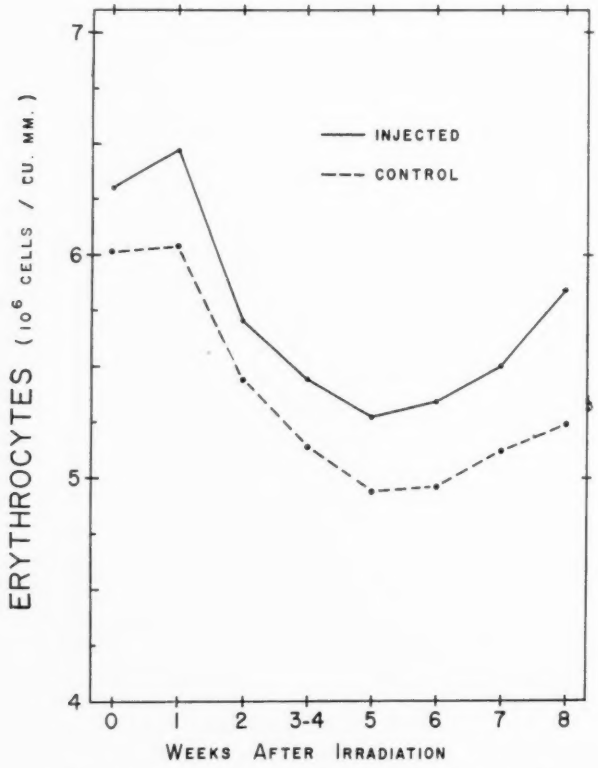

Fig. 4.—Erythrocyte counts of same animals in Fig. 1.

Rabbits Receiving Homologous Bone Marrow Emulsion Three Days after Irradiation (Group V).—Emulsion administered at this time interval after exposure resulted in the highest survival (60 per cent) of any method. Only 20.8 per cent survived thirty days or more in the comparable control group. Figs. 1 to 4 provide a comparison of the total leukocytes, neutrophils, lymphocytes, and erythrocytes with the comparable control group. No significant difference exists between the curves of the various blood elements. Furthermore, considering the neutrophils alone, there is no difference in the curves of those animals surviving and dying in this treated group (Fig. 6). The same is true for the lymphocytes. As with the control animals, we were unable to ascribe the cause of death to any specific organic lesion.

Of interest was the observation that of seven additional rabbits receiving 1,400 r total body irradiation, and then marrow emulsion three days after exposure, six survived. Four of these six received a single injection daily for three days. This group is too small to evaluate, but suggests that possibly multiple injections may be of increased benefit, particularly since the level of exposure was close to an expected 100 per cent mortality.

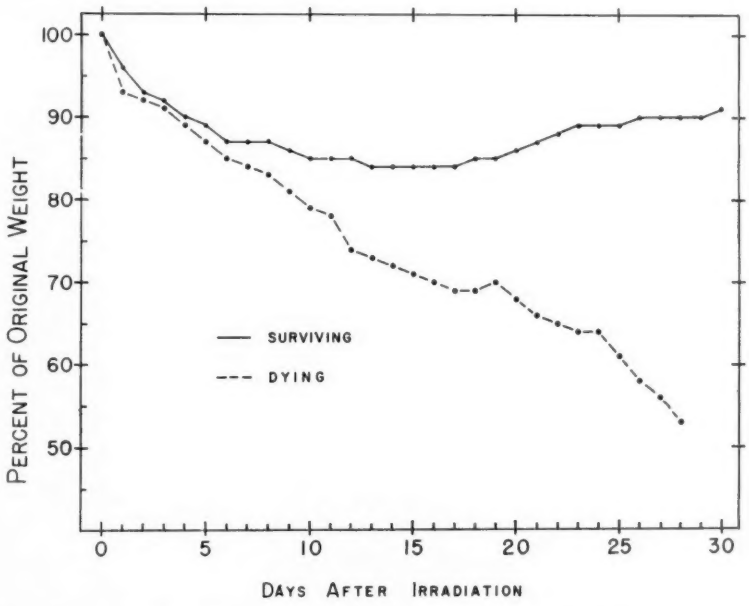

Fig. 5.—Weight curve of all surviving animals compared with that of those dying before thirty days.

Rabbits Receiving Homologous Marrow Emulsion Supernate Three Days after Irradiation (Group VI).—The question arises as to whether increased survival from the injected marrow emulsion is due to "repopulation" of the irradiated marrow by the injected cells, to elaboration of the effective substance by the injected cells in the recipient animal, or to the noncellular fraction of the emulsion. This group was studied to determine whether survival depends on "colonization" and growth of the injected marrow cells in the recipient animal. In this group, thirty-day survival was 53.3 per cent, a figure closely approaching that obtained with the whole marrow emulsion. In all other respects this group closely conformed to the findings in the latter group.

The Significance of Other Factors.—

Sex: Approximately an equal number of males and females were used in this study and were divided evenly within the various experimental groups. We did not find the sex of the animal to be a factor in determining survival, the relative proportion of males and females surviving in both treated and control groups being equal.

Weight: The average weight of the animals used was 2.5 kilograms, with a spread of less than 150 grams between groups. Survival bore no relationship to the preirradiation weight of the animal, larger animals doing no better than smaller ones. However, the weight variation between animals is too small to validly evaluate this. As indicated previously, the most significant prognostic aid was the weight curve, animals dying generally showing a progressive loss to death, whereas those surviving reached their lowest level at about two weeks and began to regain thereafter.

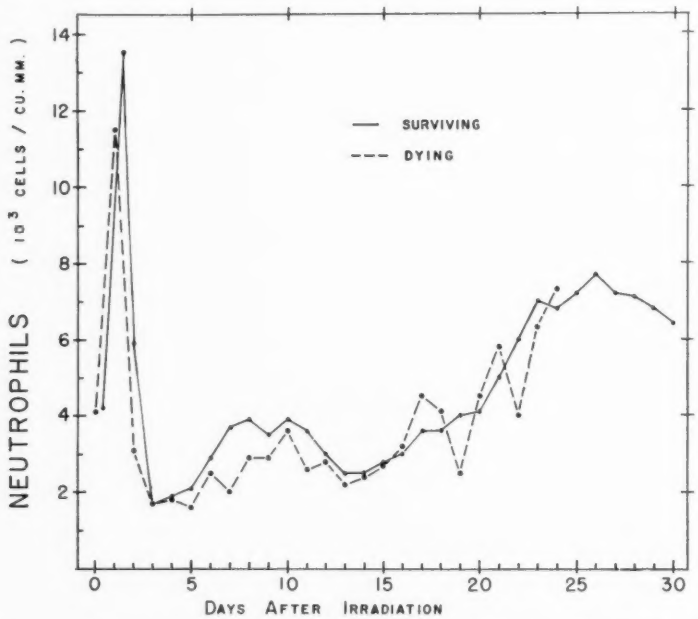

Fig. 6.—Neutrophil counts of rabbits receiving marrow emulsion three days after irradiation. Curve of surviving animals compared with that of those dying before thirty days.

Amount of material injected: Table III illustrates the average volume and number of cells injected. There is no significant difference between those animals surviving or dying in regard to number of cells injected. Other workers have obtained beneficial results on survival with relatively small amounts of material, and it does not seem that these results depend primarily on a large quantity of material being injected, but rather on source of material, time and route of administration, and possibly method of preparation.[1, 3, 4]

Prolongation of life in those animals dying: The number of animals dying at each daily interval following irradiation is given in Table IV. As mentioned previously, one-half of the control animals died within a twelve-hour period after irradiation. The other major period of mortality was toward the end of the second week after irradiation.

The median survival times for those animals dying before thirty days are as follows: controls living at least three days, 11.5 days; marrow emulsion

TABLE IV. TIME OF DEATH OF THOSE ANIMALS DYING WITHIN THE THIRTY-DAY PERIOD AFTER IRRADIATION

GROUP	DAYS AFTER IRRADIATION																												
	1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
I	29	1	6	1	2	2				1		2	9		1	1															
II											1	1	1				1		1	1							1				
III	2	2				3				1		1	2	1			1	1				1						1			
IV		1			1		1	2	2	1	1	5	2	2		1			1					1						1	
V						1		2			1	3	1			1	1		1									1			
VI								1	1			1	2	2	3	1						1	2								

three days after irradiation, 11.6 days; marrow emulsion supernate three days after irradiation, 14 days; and marrow emulsion one day after irradiation, 10.4 days. It is apparent that, although the survival rate varies significantly between certain groups, there is no evidence of prolongation of life in those animals dying before thirty days, regardless of treatment.

The median survival time for all animals dying in the control group is one day. This results from the fact that 50 per cent of the rabbits died within twelve hours of irradiation. Those animals treated within one hour of exposure, and subsequently dying before thirty days, had a median survival time of 8.5 days. This approaches the other treated groups, and is considerably longer than that of the entire control group. This suggests that immediate treatment, to some extent, is effective in counteracting the factor or factors responsible for death shortly after exposure, but not those causing death at a later period.

DISCUSSION

Survival of rabbits after 1,200 r total body irradiation was increased by the intravenous administration of homologous bone marrow emulsion and the marrow emulsion cell-free supernate. The fact that these procedures were carried out three days after exposure demonstrates that lethal effects can be altered even after this time interval. How much more this time interval can be lengthened between irradiation and treatment is not known, since the reason for increased survival is not understood. In the rabbit at least, we doubt that earlier regeneration of hematopoietic tissue is the basic factor, since we were unable to demonstrate any evidence of such from a study of the peripheral blood or marrow. Storer[5] reached the same conclusion from his study on rats. On the contrary, other investigators have demonstrated that earlier hematopoietic regeneration is associated with increased survival rates.[1, 6, 7] However, there is so much difference between species and strains of animals in response to total body irradiation that for the present this question cannot be answered satisfactorily.

Although the number of animals used is too small to evaluate, it is of interest that six of seven additional rabbits receiving 1,400 r of total body irradiation survived after the administration of marrow emulsion. Four of these six rabbits received multiple injections, suggesting that such a procedure may increase the effectiveness of treatment.

The effectiveness of the cell-free fraction of the marrow emulsion would indicate that growth, or even the presence, of injected cells in the irradiated animal is not required. Since serum alone was not effective, the factor increasing survival must be resident in the marrow itself, at least in effective quantity. The demonstration by Brecher[4] that parabiosis after exposure reduces mortality indicates that the factor is also present in the circulating blood, but under these circumstances the amount supplied to the irradiated partner would greatly exceed quantitatively that supplied by relatively small amounts of transfused serum or whole blood.

It is still not clear whether the hematopoietic cells themselves or the interstitial tissues of bone marrow are the source of the effective factor. If the hematopoietic cells of the marrow are a source, then the lymphoid tissue of the spleen and intestine and parenchymal cells of the liver must also be considered a source, since shielding of these tissues has produced beneficial results.[9] There is an alternative hypothesis that interstitial cells common to these tissues are the source, and critical experiments to determine this point have not been carried out. Storer[5] has demonstrated that by implanting the tails of rats into their peritoneal cavity, the inactive fatty marrow of the tails converts to an active cellular state. Shielding of such active marrow during total body irradiation is then effective in reducing mortality, whereas shielding of the normally fatty marrow of the tail is not. This suggests that the hematopoietic cells may be a source of the effective factor, but there are also changes in the interstitial tissues of marrow associated with increased activity that may be of equal importance.

The reason for the relative ineffectiveness of marrow given within one hour and at twenty-four hours after exposure is not clear. In the rabbit profound shock is present after total body irradiation, and undoubtedly the large number of animals dying within twenty-four hours of exposure are lost for this reason. This may explain why treatment within this time interval was not appreciably effective, since the relatively small amount of emulsion injected could hardly be expected to reverse this process. On the other hand, 50 per cent of all control animals irradiated died in the first twelve hours, whereas only 10 per cent of the rabbits treated within one hour of exposure died in this same time interval. Either treatment was to some extent effective in reducing shock, or death at this time was due to other factors than shock.

SUMMARY

Both homologous bone marrow emulsion and the supernatant fraction of the emulsion increased survival following 1,200 r of total body irradiation when injected intravenously in rabbits. This beneficial effect was observed to a significant degree only in those animals treated three days after exposure; the effect was less marked when treatment was carried out within one hour and at twenty-four hours after exposure. Homologous serum was ineffective in increasing survival.

No significant differences were observed in the daily peripheral blood counts or the marrow of irradiated animals, regardless of whether treated or not, and regardless of whether they died or survived.

It is suggested that the beneficial effect of bone marrow emulsion does not depend upon growth of the injected cells in the irradiated rabbit, but rather upon a noncellular fraction of the marrow emulsion.

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PROTEOLYTIC ENZYME STUDIES IN IRRADIATED RABBITS

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THROMBOCYTOPENIA and a humoral coagulation defect[1, 2] are factors in the hemorrhagic phenomena of the postirradiation syndrome which have been most frequently investigated. Less attention has been given to the fibrinolytic phenomena, as observed first by Cronkite in the Bikini animals[3] and later in irradiated dogs.[3] Recent work by Colgan, Gates, and Miller[4] has explored this aspect further. Since our interest was focused on changes occurring in the irradiated rabbit it seemed pertinent to investigate the fibrinolytic mechanism by (1) observing the spontaneous lysis of clots and (2) determining the serum profibrinolysin and (3) antitrypsin activity. Trypsin inhibitor was determined instead of antifibrinolysin because the methods for the latter which were tested[6, 7] were found to be unsatisfactory due to instability of fibrinolysin standard solutions. Suggestive evidence has indicated that trypsin inhibiting and fibrinolysin inhibiting substances in serum may not be identical.[5]

MATERIALS AND METHODS

Ten rabbits weighing between three to five pounds were given 900 r total body irradiation, with a 250-KV Westinghouse Quadrocondex. Each animal was irradiated separately in a cylinder of four thicknesses of 1 mm. pliafilm. They were rotated at 1.3 r.p.m. in an upright position. Focal distance was 1 meter. Filter was 0.5 mm. copper plus 1 mm. aluminum.

Blood samples were obtained every other day by cardiac puncture, using the sterile two-syringe technique. Two milliliters of blood were aspirated into the first syringe and discarded (in order to avoid contamination of the blood with tissue fluid) and the 7 milliliters withdrawn in the second syringe were used. The serum obtained after clotting and centrifugation was stored at −20° C. until used.

Borate buffer was prepared from M/20 sodium borate and M/5 boric acid—M/20 sodium chloride mixture and adjusted to an actual pH of 7.50 with M/20 sodium borate.

Streptokinase: To one vial containing 148,000 units of streptokinase and 17,000 units of streptodornase (Lederle, lot 7-1089-121A), 5 ml. of borate buffer were added (strength: 2,960 units of streptokinase in 0.1 ml.).

Thrombin: To one vial containing 5,000 National Institutes of Health units of thrombin (Parke, Davis topical bovine thrombin) 1 ml. of 0.9 per cent NaCl, and 1 ml. of glycerol were added;[6] 0.2 ml. of this mixture was added to 2.3 ml. of 0.9 per cent NaCl, so that 0.1 ml. of the final dilution contained 20 N.I.H. units of thrombin.

Fibrinogen: Five grams of bovine Fraction I (Armour) and 5 Gm. of NaCl were added to a volumetric flask containing 12.5 ml. of borate buffer and dissolved at 35° C. in double distilled water to 250 ml. Samples of 3 ml. were frozen immediately and stored at

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–20° C. Samples were thawed at 35° C. and diluted shortly before use with borate buffer to a 0.2 per cent fibrinogen solution. This was kept at 35° C. and discarded after one hour.[6] *Serum dilutions* were made $\frac{1}{10}$ to $\frac{1}{80}$, or higher, with borate buffer.

Trypsin: Five hundred thousand Armour units of crystalline trypsin (Tryptar, Armour) were dissolved in a 500 ml. volumetric flask containing N/400 HCl and stored as a stock solution at 4° C. Final dilutions in borate buffer containing 65 Armour units of trypsin in 0.5 ml. were freshly prepared before each determination. This amount of trypsin was inhibited by 10 micrograms of crystalline soybean trypsin inhibitor (Worthington Biochemical Laboratory), and contained therefore 10 micrograms of active trypsin.[8]

Serum profibrinolysin was determined in duplicate in a water bath at 35° C. The method of Christensen[8] was used, with minor modifications. Portions of 0.4 ml. of diluted rabbit serum were preheated in the water bath for five minutes, then activated for ten minutes by addition of 0.1 ml. of a streptokinase solution containing 2,960 units of streptokinase. During the activation period 0.1 ml. of a thrombin solution, containing 20 N.I.H. units of thrombin was added. After ten minutes, 0.6 ml. of a 0.2 per cent fibrinogen solution was added to each tube, which was shaken briskly once. Thirty minutes later the clots were inspected for lysis. The highest dilution (1/a) of serum giving lysis of the clot in thirty minutes was considered to represent one unit of profibrinolysin. Results were expressed as units of profibrinolysin per milliliter of serum. Duplicate results were averaged. This unit of rabbit profibrinolysin was arbitrary, and not equivalent to a unit of human profibrinolysin,[9] since different amounts of activator were used.*

Serum antitrypsin was determined in duplicate in a water bath at 35° C., following Christensen's[8] method. Portions of 0.5 ml. of diluted rabbit serum were preheated in the water bath for five minutes, after which 0.5 ml. of the trypsin solution (containing 65 Armour units or 10 micrograms of trypsin) was added. Exactly *five* minutes later 1 ml. of a 0.2 per cent fibrinogen solution was pipetted into each tube. Ten minutes after the addition of fibrinogen 0.2 ml. of a thrombin solution (containing 40 N.I.H. units of thrombin) was added. The tubes were shaken briskly once. Ten minutes after the addition of thrombin the clots were inspected for lysis. The highest serum dilution (1/a) in which the clot was still present was considered to contain serum inhibitor equivalent to 10 micrograms of trypsin. Inhibitor units were expressed as the number of micrograms of active trypsin inhibited by 1 ml. of serum. Duplicate results were averaged.†

Spontaneous lysis time: One milliliter of rabbit blood obtained by cardiac puncture was added to each of two sterile tubes of 11 mm. internal diameter. These were stoppered with sterile paraffined corks. The tubes were incubated in a water bath at 35° C. and

TABLE I. PROFIBRINOLYSIN (UNITS PER MILLILITER) IN IRRADIATED RABBITS (900 r)

RABBIT NUMBER	CONTROL VALUES:		POSTIRRADIATION DAYS:						
	1	2	1	2	3	4	5	6	7
1 H	100	100	100		150		150	†450*	
3 H	100	100	100		200	†			
9 H	200	250	350		450	†			
10 H	150	150	†						
11 H	100	100	100		150		325	†	
15 H	175	200	†						
16 H	100	100			200		†		
17 H	100		150		100		125		200†
19 H	275	300			500	†800*			
22 H	125	150	†						

*Postmortem sample.
†Died.

**Calculation:* 0.4 ml. of serum dilution (1/a) contains 1 unit of profibrinolysin, thus the undiluted serum contains a $\times \frac{10}{4}$ units of profibrinolysin per ml.

†*Calculation:* 0.5 ml. of serum dilution (1/a) contains inhibitor units equivalent to 10 micrograms of trypsin, thus 1 ml. of undiluted serum contains inhibitor units equivalent to a $\times$ 2 $\times$ 10 micrograms of trypsin = 20a inhibitor units.

inspected daily for lysis of the clots. Clot formation and retraction were observed by inspection. Tubes were discarded after fourteen days.

Red blood cell counts and *platelet counts* (modified Dameshek's method) were determined on ear vein blood on the same days that cardiac punctures were performed.

RESULTS

Of the ten rabbits, three died immediately after irradiation (Tables I and II). Of the remaining seven rabbits, three died on the fourth postirradiation day, one on the fifth, two on the sixth, and one on the seventh postirradiation day.

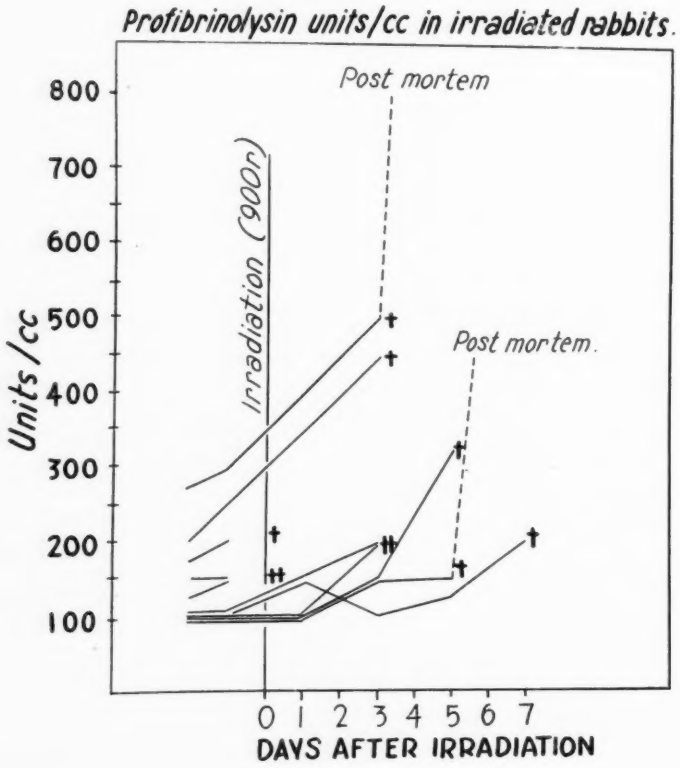

Fig. 1.

Four of the rabbits showed dehydration at autopsy. Three of these showed signs of coccidial liver abscesses.* The dehydration was not reflected in the gradually decreasing red cell counts in three of these animals. Two rabbits (1H and 17H) with terminal thrombocytopenia (53,000 and 40,960) and anemia (R.B.C. 2.65 million) died of traumatic hemopericardium without other signs of bleeding shortly after cardiac puncture, on the sixth and seventh postirradiation days. The other rabbits showed neither thrombocytopenia nor gross hemorrhages at autopsy. Clot formation and retraction as observed by inspection were not grossly impaired.

*A frequently occurring disease in North American laboratory rabbits.

In all of the seven rabbits available for postirradiation study, the serum profibrinolysin (ranging 100 to 300 units per milliliter before irradiation) increased to a final range of 150 to 500 units per milliliter after irradiation (Table I, Fig. 1). The trypsin inhibitor (ranging 400 to 600 units per milliliter before irradiation) was also increased in all rabbits to a final range of 600 to 1000 units per milliliter after irradiation (Table II, Fig. 2). On two occasions samples were taken of the unclotted inferior vena cava blood a few hours post mortem. The sera, after clotting, showed the highest profibrinolysin (450 and 800 units per milliliter) and antitrypsin (1,200 units per milliliter) content in the series. The spontaneous lysis times were not enhanced after irradiation. No lysis was observed after fourteen days.

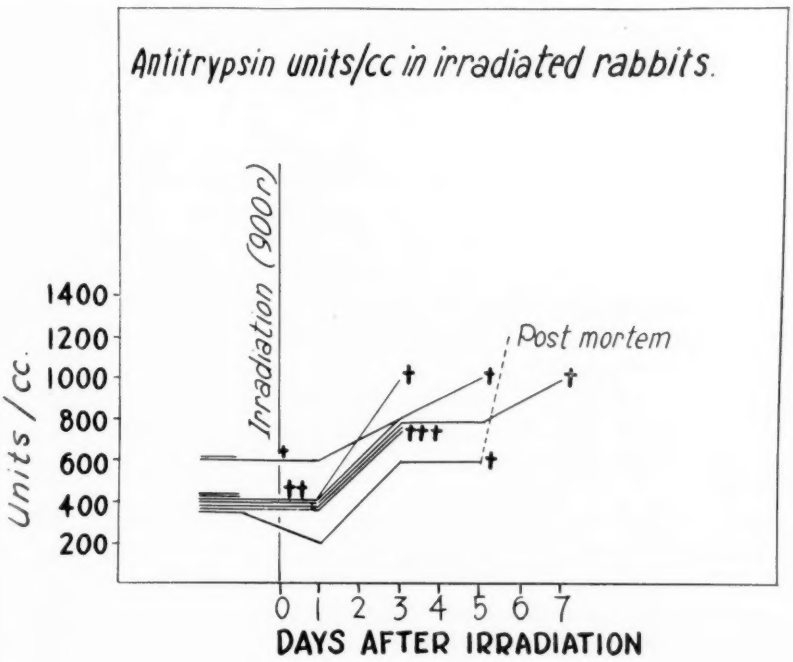

Fig. 2.

DISCUSSION

Activation of serum profibrinolysin in rabbits required a minimum of 2,960 Lederle units of streptokinase (twenty times the amount used in human beings). The resulting profibrinolysin values of normal rabbit serum were from 100 to 300 units per milliliter. In other experiments human profibrinolysin values after activation with 148 Lederle units of streptokinase ranged from 6,250 to 13,750 units per milliliter.[9] Cliffton[10] and Johnson[11] also reported that rabbit serum contained less profibrinolysin than human serum. This is due to a smaller profibrinolysin content of rabbit serum and not to incomplete activation, since very little difference has been found between the amount of proteolytic activity in rabbit serum activated either by streptokinase, protamine, or

TABLE II. ANTITRYPSIN (UNITS PER MILLILITER) IN IRRADIATED RABBITS (900 r)

RABBIT NUMBER	CONTROL VALUES:		POSTIRRADIATION DAYS:						
	1	2	1	2	3	4	5	6	7
1 H	400	400	200		600		600	†1200*	
3 H	400	400	400		800	†			
9 H	400	400	400		1000	†			
10 H	400	400	†						
11 H	600	600	600		800		1000	†	
15 H	400	400	†						
16 H	400	400	400		800		†		
17 H	400		400		800		800		1000†
19 H	400	400	400		800	†			
22 H	600	600	†						

*Postmortem sample.
†Died.

spontaneously.[10] In these and in other experiments in this Laboratory normal rabbit serum contained less antitrypsin (400 to 600 units per milliliter) than human serum (800 to 1,800 units per milliliter).[9] Guest and associates[6] found rabbit antibovine—fibrinolysin higher than human antibovine—fibrinolysin. Christensen[5] gave only one value for rabbit antitrypsin which was lower than the human average. According to Storey, Wish, and Furth[12] the plasma volume in rabbits irradiated with 1,000 r decreases slowly after irradiation, reaching 15 to 20 per cent decrease at the eighth postirradiation day. The changes in serum profibrinolysin and antitrypsin in these experiments cannot be solely explained on changes in plasma volume, since in most instances the values were already doubled after the third irradiation day. The marked elevation of serum profibrinolysin and antitrypsin in the postmortem samples was probably part of the terminal picture. Colgan, Gates, and Miller[4] reported an increase in serum profibrinolysin and urinary fibrinolytic activity in irradiated dogs. They related the urinary changes to pre-existing pulmonary hemorrhages. No determinations of urinary fibrinolytic activity were done in the rabbit experiments here. Rosenthal and Benedek[13] observed relatively inconspicuous hemorrhage in rabbits following intense total body irradiation in contrast to dog, guinea pig, and man. In our observations serum profibrinolysin rose in the absence of spontaneous gross hemorrhage and therefore cannot be explained by the occurrence of hemorrhage. No accelerated lysis of whole blood clots was observed, in contrast to the observations in irradiated goats and pigs (Cronkite)[1] and in irradiated dogs (Jackson and associates).[3] Therefore this study is inconclusive as to whether fibrinolysis in rabbits contributes to postirradiation hemorrhage. It may be concluded, however, that in irradiated rabbits serum profibrinolysin and antitrypsin may rise in the absence of spontaneous hemorrhage and spontaneous rapid clot lysis.

SUMMARY AND CONCLUSIONS

In a series of irradiated rabbits serum profibrinolysin and antitrypsin increased after total body irradiation. Spontaneous rapid lysis of clots was not observed. Thus, in irradiated rabbits a rise in serum profibrinolysin and antitrypsin may occur in the absence of spontaneous hemorrhage or spontaneous lysis.

The technical assistance of Mrs. M. B. Munford and Miss Frances Chubb is highly appreciated. We are indebted to Dr. George Y. Shinowara for the determination of the fibrinogen content of Armour's bovine Fraction I. We are also grateful to Mr. Alvinza E. Bunner, Dr. Bertha A. Bouroncle, Mrs. Helen Chrysler, Mr. Cyrus Green, and Mrs. Evelyn Bruce. "Tryptar" was kindly supplied by Dr. M. C. Ziporyn of Armour Company, Chicago, and streptokinase-streptodornase by Lederle Laboratories Division, American Cyanamid Company, New York, through the courtesy of Dr. J. M. Ruegsegger.

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A STUDY OF ANTIGEN LOCALIZATION AND DEGRADATION AND THE HISTOLOGIC REACTION IN THE SPLEEN OF NORMAL, X-IRRADIATED, AND SPLEEN-SHIELDED RATS

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IT HAS been established that total body x-irradiation markedly inhibits humoral antibody concentration following the single injection of an antigen given shortly after irradiation.[1] Jacobson and associates,[2] using the rabbit, have shown that lead shielding of the spleen during irradiation markedly decreases the inhibitory effect of irradiation upon antibody formation. This observation has been confirmed in the rat.[3] The protective effect of spleen shielding was observed even though splenectomy was performed twenty-four hours before the injection of antigen.[4] This latter phenomenon can be observed in the rat if the antigen is given intraperitoneally.[3]

Both the cellular sites and the biochemical mechanisms of antibody synthesis are in doubt.[5] Furthermore, the mechanisms by which antibody formation is depressed following total body x-irradiation and is protected by spleen shielding are still obscure.[1, 6] The experiments reported here represent a study of certain traceable steps of antigen localization and degradation and of antibody formation in the normal, irradiated, and spleen-shielded rat with particular reference to the spleen. Their purpose was to discover in what ways total body x-irradiation may alter the antibody forming mechanism.

The questions considered were as follows: does total body x-irradiation interfere with rate of removal of antigen from the blood stream (phagocytosis), with tissue distribution of a particulate antigen following a single intravenous injection (localization), or with the rate of disappearance of antigen (digestion)? Is the histologic reaction to a single antigenic stimulus altered by total body x-irradiation?

MATERIALS AND METHODS

Experimental Plan.—In each of three experiments, a similar plan was followed. In the first experiment, one group of rats was given 500 r total body x-irradiation and one was retained as a normal group. Both groups were injected intravenously twenty-four hours later with a standard dose of killed *Salmonella typhi* organisms labeled with I^{131} and with a standard dose of sheep erythrocytes. Three rats of each group were bled at intervals of five, fifteen, thirty, and sixty minutes after injection to study the rate of antigen disappearance from the blood stream. Three animals from each group were sacrificed at

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fifteen and sixty minutes and at one day, four days, and six days after injection to determine the amount of labeled antigen in various organs and to obtain tissues for radioautographs and for histologic study. Serum for determining antibody concentrations was collected from the rats sacrificed on days 4 and 6. Three animals from each group were maintained in metabolism cages throughout the experiment to determine the rate of urinary excretion of the I^{131}. Noninjected rats, both irradiated and nonirradiated, were sacrificed in each experiment.

The second experiment differed from the first in that NaI^{131} and noniodinated *Salmonella typhi* vaccine were injected separately in order that the fate of unattached NaI^{131} might be compared with that of I^{131}-labeled vaccine.

The third experiment was similar to the first, except that rats with lead shielding of the spleen during irradiation were also studied.

Animals and Care.—Young adult male Sprague Dawley rats weighing from 150 to 240 grams were used throughout the study, distributed by random selection to form the experimental groups.

The rats were changed from Purina Laboratory Chow to a synthetic, liquid diet five days before the beginning of experimental procedures. This largely prevented contamination of urine by food and facilitated the addition of iodine to the ration. The synthetic diet was essentially the 4C diet used by Wissler and collaborators[7] with dextrin substituted for cornstarch and the Brewer's yeast replaced with additional vitamins in the quantity known to be contained in the yeast usually added. The liquid diet was prepared by mixing 20 Gm. of diet powder per animal per day with an equal volume of tap water. To prevent localization of I^{131} in the thyroid gland, Lugol's solution was added to the diet and to the drinking water in the amount of 0.5 ml. Lugol's solution per 100 ml. diet or tap water.

Antigen Preparation and Antibody Determination.—Washed sheep erythrocytes and formalin-killed *Salmonella typhi* organisms (Hopkin's strain)* were used as antigens. The general methods of preparing the antigens, bleeding the animals, and measuring the hemolysin and agglutinin content of the sera have been summarized by Cannon and coworkers.[8, 9] For titrations, the double dilution method was employed starting with a serum dilution of 1:15 for hemolysin determinations and 1:10 for agglutinin titration. The last tube showing complete hemolysis was selected as the end-point for the hemolysin determination. Noniodinated *Salmonella typhi* organisms were used as antigen in the agglutinin determinations.

Iodination.—The *Salmonella typhi* organisms were labeled with $iodine^{131}$ using a modification of the method of Pressman.[10] The reaction was performed at room temperature. To 5.0 ml. of carrier-free potassium $iodide^{131}$ (about 20 millicurie)† were added 0.5 ml. of 0.01M KI, 5 drops of 1M $NaNO_2$, and 1.0 ml. of 2.5 N HCl. The solution turned slightly yellow. After five minutes, 1.0 ml. of 2.5 N NaOH was added, followed immediately by 10 ml. of a concentrated suspension of formalin-killed *S. typhi* organisms. The mixture was allowed to stand for about forty minutes. The reaction vessel was kept stoppered throughout the entire procedure to minimize contamination of the air. The unattached radioiodine was removed with four 50 ml. washings with distilled water, and the organisms were suspended in 50 ml. of sterile saline.

This method of iodination gave 29,000 and 28,100 counts per gamma of antigen nitrogen on two different preparations. The preparation used in experiment III was prepared with the same amount of antigen but only one-fifth the amount of the iodination mixture. This preparation contained 2,460 counts per gamma of antigen nitrogen.

After the four washings between 95 and 98 per cent of the radioactivity was attached to the organisms. Incubation of the labeled antigen for seven days at 4° and 37° C. in rat serum, buffered saline, and in acetate buffer at pH 4 caused no appreciable

*Obtained through the courtesy of Mr. Ross Benham, Director Clinical Microbiology Laboratory, Albert Merritt Billings Hospital, Chicago, Ill.

†Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

increase in radioactivity of the supernatant. Incubation at the same temperatures, with distilled water and boric acid-KCl-NaOH buffer at pH 10, resulted in a marked increase in radioactivity in the supernatant.

Serological tests established that this method of iodination of *Salmonella typhi* did not alter appreciably the ability of the organisms to agglutinate with rabbit anti-*Salmonella typhi* sera.

Measurement of Radioactivity.—Determinations of radioactivity were made using a Welch-Allen well type gamma counter. Weighed tissues were placed in a standard 16 by 150 mm. test tube. Since gamma radiation was measured, correction for self-absorption and for absorption by the glass were not made. Geometry was maintained constant.

A standard was prepared for each experiment using one-tenth of the injected radioactivity. Activity of this standard was determined on each day that counting was done. The results of activity determined on any given day were then expressed in terms of per cent of injected activity per total organ and per gram of tissue.

Dosimetry and Spleen Shielding.—The x-rays were generated in a 250 kilovolt machine operating at 15 milliamperes.* A 0.5 mm. copper filter and a 1.0 mm. aluminum filter were used. The dose rate averaged 30 r per minute at 75 cm. A single dose of 500 r per rat was used in the experiments. In experiment III, four animals with spleen exteriorized were irradiated simultaneously with four animals having lead shielding of the spleen.

The method of spleen shielding was similar to that used by Jacobson and associates for rabbits and mice.[2, 11] In experiment III, the irradiated animals and normal animals were sham-operated. Precautions to avoid infection were observed in all cases.

Histotechnique.—Histologic material was fixed both in Carnoy's alcohol-acetic acid-chloroform mixture and in 10 per cent formol-saline. Tissues were imbedded in paraffin. Methyl green-pyronin stains were made on the Carnoy fixed material using the Popper modification of the Unna-Pappenheim procedure.[12] Azure B stains were made on Carnoy fixed material according to Flax and Himes.[13]

To demonstrate the specificity of these stains for ribonucleic acid, sections were treated for forty-five minutes at room temperature with a 0.02 per cent solution of crystalline ribonuclease.† Control sections mounted on the same slide were treated with an identical solution which did not contain ribonuclease. The ribonuclease was treated as directed by Flax and Himes[13] to destroy proteolytic enzymes with which commercial crystalline ribonuclease is frequently contaminated.[14] It should be pointed out that ribonucleic acid is soluble in many of the commonly used buffer systems.[15] Hence, rigid control of the digestion procedure must be employed.

Radioautographs were prepared by mounting paraffin imbedded sections in the dark on Eastman Nuclear Track Plates type NTB with 10 to 15 μ emulsion. After one to four weeks exposure, the sections were deparaffinized, and the emulsion was developed in Eastman D-19 developer and fixed in 25 per cent sodium thiosulfate. The tissues were lightly stained with hematoxylin and eosin. The autographs demonstrated only protein-bound iodine, presumably bound to antigenic material. Iodide and nonprotein-bound iodine were washed out in the preparation of the histologic material.

Attempts to demonstrate the *S. typhi* organisms in the tissues of animals sacrificed as soon as fifteen minutes after injection of antigen were unsuccessful.

RESULTS

Experiment I.—Twenty-four hours after x-irradiation, all animals excepting one in each group were given 1.0 ml. of I^{131}-labeled typhoid vaccine (about 500,000 counts per minute) and 0.5 ml. of a 0.5 per cent suspension of washed

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†Lot number 381-059. Obtained through the courtesy of Dr. Howard Glass, Research Division, Armour and Company, Chicago, Ill.

sheep erythrocytes. The animals were treated as outlined in *Materials and Methods*. Radioactivity in the liver, spleen, kidneys, lungs, thyroid, mesenteric lymph nodes, and femurs was determined. Small portions of the liver, spleen, kidney, lung, and lymph node were taken for histologic examination.

The per cent of injected activity in the spleen, liver, lung, blood, and urine is shown in Table I and Fig. 1. Antibody titers are recorded in Table IV. The kidneys never contained more than 0.5 per cent of the injected activity. Only 0.04 per cent of the injected radioactivity was found in the thyroid and lymph nodes and 0.15 per cent in both femurs sixty minutes after injection. Preliminary experiments showed that the amount of activity did not increase in these organs at any later time; therefore, the activity in these organs was not determined in all animals. Activity of the feces was not determined, since preliminary experiments showed that only about 5 per cent of the injected activity was ever eliminated by this route.

It is evident that there were no marked differences between normal and x-irradiated animals in the distribution of radioactivity at any time after antigen injection. There appeared, therefore, to be no difference between the two groups in the rate of phagocytosis, in the tissue distribution of the antigen, or in the disappearance of antigen from the tissues.

About 65 per cent of the injected activity was found in the liver and 15 per cent in the spleen fifteen minutes after antigen injection in both groups. When the radioactivity was expressed in terms of per cent injected activity per gram of tissue at fifteen minutes after injection, the liver contained 5.5 per cent per gram in both groups. The spleen, on the other hand, contained 38 per cent per gram in the irradiated animals and 20 per cent per gram, in the normal animals. The spleens of irradiated animals were much smaller than those of the normal rats due to the destructive effect of irradiation upon lymphocytic tissue. Apparently the phagocytic cells of the spleen had ingested about the same amount of antigen in both groups.

The curve of the disappearance of radioactivity from the blood stream showed a rapid removal of the radioactive particles by the reticuloendothelial system. The high levels of activity in the liver and spleen support this classic concept.

Rapid degradation of a large portion of the antigen was suggested by the rapid urinary excretion of radioactivity. At the end of the first twenty-four hours after antigen injection, 47 per cent of the activity had been excreted by the irradiated animals and 68 per cent by the control animals. This difference in percentage excretion remained rather marked until the fourth day after injection. The reason for this difference was not apparent since there was no difference in tissue distribution in the organs counted to explain it. It may be related to general effects of irradiation which reduce food and fluid intake. The total excretion at the end of the sixth day was the same in both groups.

About 89 per cent of the injected activity had been measured in the control animals on the sixth day after injection, and about 87 per cent in the irradiated animals. Some of the remainder was presumably excreted in feces, perspiration, and exhaled air, and some was present in the tissues which were not counted.

TABLE I. RADIOACTIVITY IN SELECTED ORGANS, URINE, AND BLOOD FOLLOWING INTRAVENOUS INJECTION OF I^{131}-LABELED TYPHOID VACCINE INTO NORMAL AND IRRADIATED RATS

EXPERIMENT I

TIME AFTER INJECTION	PER CENT OF INJECTED RADIOACTIVITY					
	TOTAL ORGAN			GRAM OF TISSUE		
	LIVER	SPLEEN	LUNG	LIVER	SPLEEN	LUNG
	Normal					
15 minutes	69 ± 2.7*	16 ± 0.9	1.3 ± 0.12	5.9 ± 0.41	20 ± 2.1	1.0 ± 0.13
60 minutes	63 ± 2.1	16 ± 2.3	1.2 ± 0.88	5.7 ± 0.45	20 ± 2.4	0.82 ± 0.023
1 day	7.5 ± 0.50	3.2 ± 0.75	0.26 ± 0.032	0.58 ± 0.070	3.7 ± 0.64	0.21 ± 0.027
4 days	3.5 ± 0.47	1.9 ± 0.57	0.076 ± 0.0167	0.35 ± 0.057	1.0 ± 0.00	0.061 ± 0.0129
6 days	3.8 ± 0.20	1.7 ± 0.21	0.052 ± 0.0033	0.35 ± 0.044	1.8 ± 0.25	0.041 ± 0.0022
	Irradiated					
15 minutes	64 ± 0.6	15 ± 0.9	1.3 ± 0.19	5.2 ± 0.26	38 ± 7.6	1.1 ± 0.07
60 minutes	62 ± 2.4	16 ± 0.3	1.0 ± 0.20	5.6 ± 0.20	40 ± 3.5	0.74 ± 0.195
1 day	8.3 ± 0.35	3.0 ± 0.53	0.46 ± 0.055	0.76 ± 0.081	7.9 ± 1.59	0.39 ± 0.066
4 days	2.7 ± 0.91	2.3 ± 0.27	0.031 ± 0.0047	0.25 ± 0.037	7.7 ± 0.89	0.023 ± 0.0040
6 days	3.6 ± 0.75	2.2 ± 0.39	0.037 ± 0.0105	0.41 ± 0.135	10 ± 2.0	0.029 ± 0.0052

EXPERIMENTAL GROUPS	PER CENT OF INJECTED RADIOACTIVITY					
	URINE DAYS AFTER INJECTION			BLOOD MINUTES AFTER INJECTION		
	DAY 1	DAY 4	DAY 6	5 MINUTES	15 MINUTES	60 MINUTES
Normal	68 ± 7.3	81 ± 7.0	83 ± 6.7	1.6 ± 0.29	0.54 ± 0.005†	0.25 ± 0.030†
Irradiated	47 ± 8.9	78 ± 5.0	81 ± 4.7	1.8 ± 0.15	0.81 ± 0.097	0.30 ± 0.040

*All results are expressed as the mean of results from three animals and the standard error unless otherwise noted.
†The mean of results from two animals and the standard error.

The second experiment was then performed to determine whether the distribution of radioactivity was due to the I^{131}-labeled *S. typhi* organisms or to the radioiodine itself.

Experiment II.—The general plan of this experiment was identical with that of Experiment I. It differed in that the radioiodide and *S. typhi* vaccine

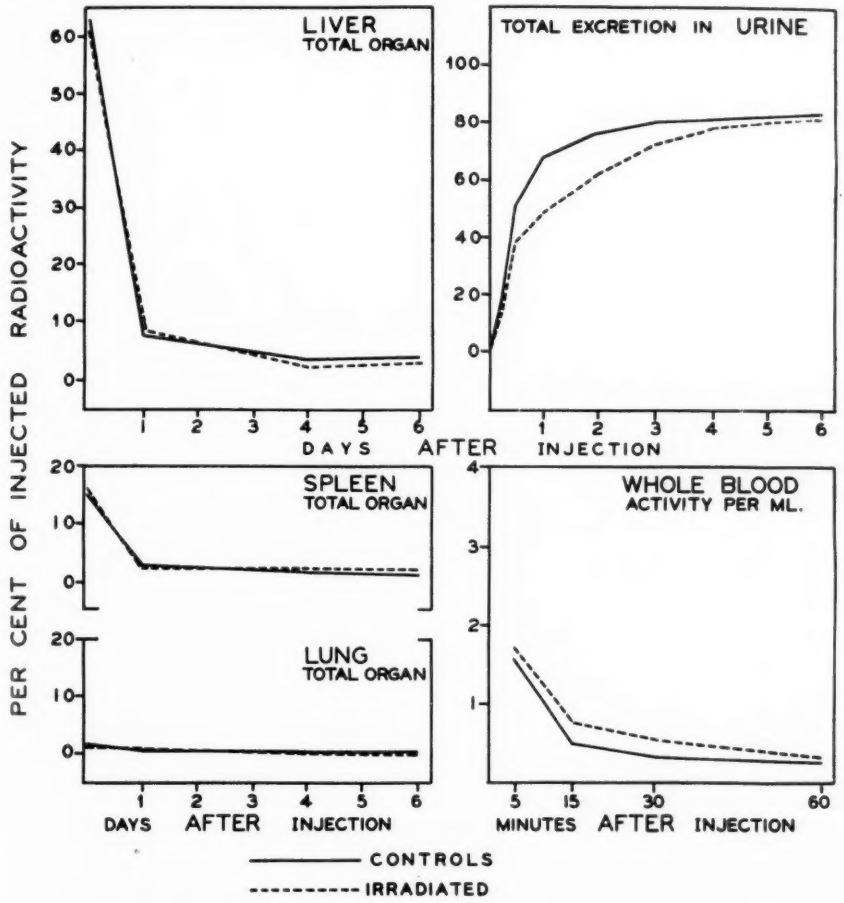

Fig. 1.—Experiment I. Radioactivity in selected organs, urine, and blood following intravenous injection of I^{131}-labeled typhoid vaccine into normal and irradiated rats. Each point is the average of three determinations.

were injected separately. Twenty-four hours after x-irradiation, 0.5 ml. of sodium iodide^{131} (about 500,000 counts per minute), 1.0 ml. formalin-killed *S. typhi* organisms, and 0.5 ml. of a 0.5 per cent suspension of sheep erythrocytes were given intravenously. The animals were treated and sacrificed in the same manner as in Experiment I. However, the thyroid, lymph nodes, and femurs were not taken for determination of radioactivity.

The results of the determinations of radioactivity in the liver, spleen, lung, urine, and blood are shown in Table II and Fig. 2. The antibody titers are recorded in Table IV.

TABLE II. RADIOACTIVITY IN SELECTED ORGANS, URINE, AND BLOOD FOLLOWING INTRAVENOUS INJECTION OF NaI^{131} INTO NORMAL AND IRRADIATED RATS

EXPERIMENT II

TIME AFTER INJECTION	PER CENT OF INJECTED RADIOACTIVITY					
	TOTAL ORGAN			GRAM OF TISSUE		
	LIVER	SPLEEN	LUNG	LIVER	SPLEEN	LUNG
Normal						
15 minutes	2.9 ± 0.32*	0.14 ± 0.015	0.46 ± 0.015	0.24 ± 0.019	0.23 ± 0.012	0.41 ± 0.026
60 minutes	2.2 ± 0.09	0.14 ± 0.006	0.38 ± 0.004	0.20 ± 0.012	0.22 ± 0.009	0.33 ± 0.012
1 day	1.7 ± 0.02†	0†	0.061 ± 0.0052†	0.010 ± 0.0055†	0†	0.039 ± 0.0029†
4 days	0	0	0	0	0	0
6 days	0	0	0	0	0	0
Irradiated						
15 minutes	3.0 ± 0.26	0.097 ± 0.0037	0.50 ± 0.047	0.23 ± 0.023	0.24 ± 0.024	0.44 ± 0.028
60 minutes	2.2 ± 0.12	0.060 ± 0.0096	0.47 ± 0.009	0.19 ± 0.000	0.17 ± 0.024	0.35 ± 0.007
1 day	1.0 ± 0.79	0.037 ± 0.0366	0.18 ± 0.116	0.10 ± 0.083	0.13 ± 0.126	0.11 ± 0.069
4 days	0	0	0	0	0	0
6 days	0	0	0	0	0	0

EXPERIMENTAL GROUPS	PER CENT OF INJECTED RADIOACTIVITY					
	URINE DAYS AFTER INJECTION			BLOOD MINUTES AFTER INJECTION		
	DAY 1	DAY 4	DAY 6	5 MINUTES	15 MINUTES	60 MINUTES
Normal	87 ± 1.8	93 ± 1.8	93 ± 2.1	1.0 ± 0.25	0.77 ± 0.073	0.61 ± 0.058
Irradiated	76 ± 6.2	96 ± 4.7	98 ± 4.7	0.82 ± 0.005†	0.99 ± 0.205	0.65 ± 0.025

*All results are expressed as the mean of results from three animals and the standard error unless otherwise noted.
†The mean of results from two animals and the standard error.

There was a striking difference in the initial tissue distribution between Experiments I and II as can be seen by comparing Table I and Fig. 1 with Table II and Fig. 2. When the radioiodine was attached to *S. typhi* organisms, 87 per cent of the injected activity in the control animals and 80 per cent of the injected activity in the irradiated animals was present in the liver, spleen, kidneys, and lungs sixty minutes after injection, about the time when greatest activity was present in the tissues. When radioiodide was given unattached to

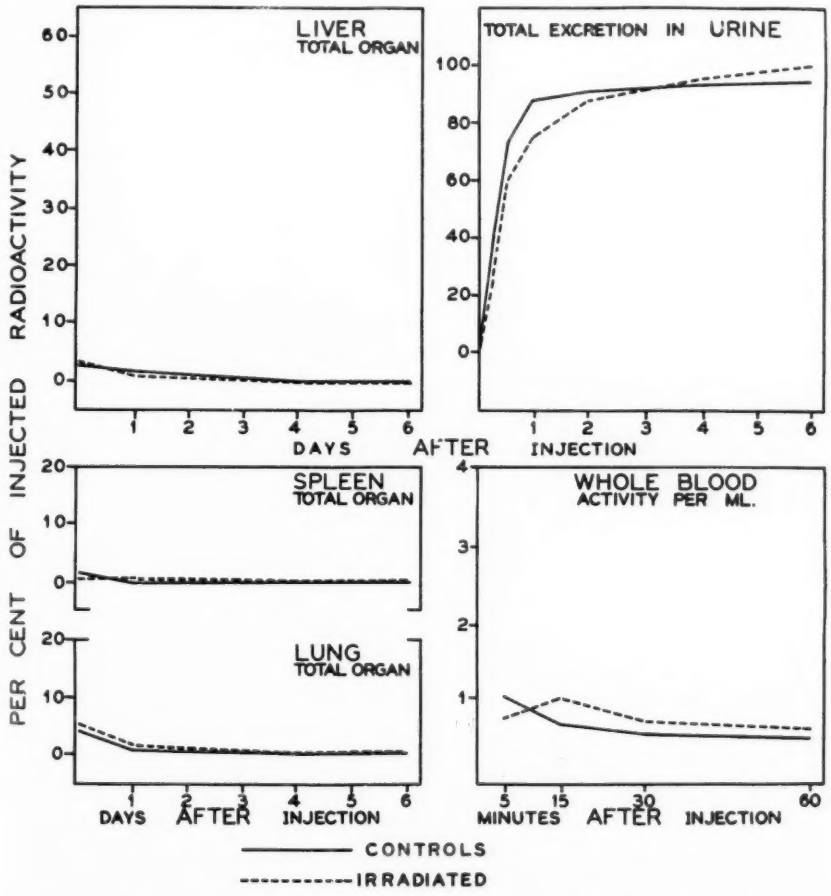

Fig. 2.—Experiment II. Radioactivity in selected organs, urine, and blood following intravenous injection of NaI^{131} into normal and irradiated rats. Each point is the average of three determinations.

S. typhi organisms, only 4.8 per cent of the injected activity in the control animals and 4.3 per cent of the injected activity in the irradiated animals was present in the liver, spleen, kidneys, and lungs sixty minutes after injection. These data are recorded in Table V.

The level of activity was between 0.2 and 0.5 per cent per gram of tissue in all organs. Thus, the amount of reticuloendothelial tissue did not determine the level of activity as it did in Experiment I.

TABLE III. RADIOACTIVITY IN SELECTED ORGANS, URINE, AND BLOOD FOLLOWING INTRAVENOUS INJECTION OF I^{131}-LABELED TYPHOID VACCINE INTO NORMAL, IRRADIATED, AND SPLEEN-SHIELDED RATS

EXPERIMENT III

TIME AFTER INJECTION	PER CENT OF INJECTED RADIOACTIVITY					
	TOTAL ORGAN			GRAM OF TISSUE		
	LIVER	SPLEEN	LUNG	LIVER	SPLEEN	LUNG
	Normal					
60 minutes	48 ± 3.5*	9.1 ± 0.52	7.1 ± 1.10	6.2 ± 1.04	16 ± 1.2	6.1 ± 0.92
1 day	9.8 ± 0.75	0.91 ± 0.146	3.7 ± 1.19	1.2 ± 0.20	1.4 ± 0.38	3.4 ± 0.92
4 days	2.5 ± 0.12	0.60 ± 0.075	0.69 ± 0.007	0.29 ± 0.032	0.44 ± 0.078	0.58 ± 0.053
6 days	2.2 ± 0.19	0.54 ± 0.047	0.12 ± 0.152	0.27 ± 0.051	0.85 ± 0.126	0.88 ± 0.232
	Irradiated					
60 minutes	54 ± 1.4	6.2 ± 0.32	9.1 ± 1.96	7.8 ± 0.50	22 ± 5.0	7.6 ± 0.92
1 day	11 ± 0.4	1.1 ± 0.26	6.3 ± 1.46	1.3 ± 0.06	3.3 ± 0.75	4.9 ± 0.78
4 days	3.1 ± 0.06†	0.72 ± 0.066†	5.6 ± 2.72†	0.29 ± 0.006†	1.7 ± 0.68†	5.0 ± 1.70†
6 days	2.2 ± 0.36	0.67 ± 0.176	0.69 ± 0.513	0.25 ± 0.065	2.1 ± 0.29	0.62 ± 0.196
	Spleen-Shielded					
60 minutes	54 ± 2.5	7.6 ± 1.71	5.8 ± 1.96	6.4 ± 0.72	15 ± 1.3	10 ± 2.47
1 day	10 ± 0.3†	1.5 ± 0.17†	5.5 ± 1.27†	1.4 ± 0.04†	2.4 ± 0.15†	5.2 ± 1.06†
4 days	4.5 ± 1.22	0.91 ± 0.197	1.8 ± 0.28	0.50 ± 0.135	2.1 ± 1.56	1.8 ± 0.17
6 days	3.1 ± 0.04	0.80 ± 0.086	1.4 ± 0.26	0.24 ± 0.015	1.1 ± 0.15	1.4 ± 0.25

EXPERIMENTAL GROUPS	PER CENT OF INJECTED RADIOACTIVITY					
	URINE DAYS AFTER INJECTION			BLOOD MINUTES AFTER INJECTION		
	DAY 1	DAY 4	DAY 6	5 MINUTES	15 MINUTES	60 MINUTES
Normal	59 ± 3.8	75 ± 1.9	77 ± 1.5	2.5 ± 0.27	2.6 ± 0.33	1.6 ± 0.00†
Irradiated	57 ± 2.6	73 ± 1.5	75 ± 1.5	1.3 ± 0.16	1.2‡	0.86 ± 0.017†
Spleen-shielded	63 ± 2.4	79 ± 2.6	81 ± 2.4	0.67‡	1.0 ± 0.04†	1.0 ± 0.03†

*All results expressed as the mean of results from three animals and the standard error unless otherwise noted.
†The mean of results from two animals and the standard error.
‡Results from one animal.

There was no marked difference between the normal and irradiated animals in the decrease of radioactivity of the blood stream immediately following injection. The decline was more gradual and the level was never as high as in Experiment I. This probably indicated that the unattached radioiodide diffused rapidly into the extravascular fluids.

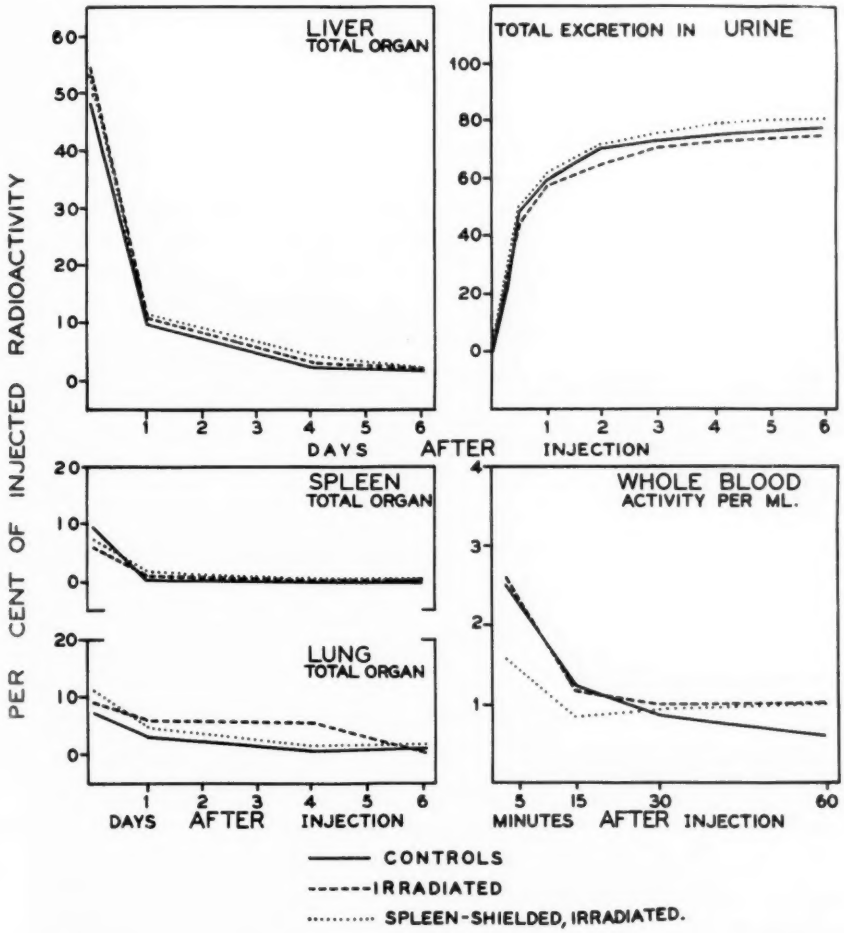

Fig. 3.—Experiment III. Radioactivity in selected organs, urine, and blood following intravenous injection of I^{131}-labeled typhoid vaccine into normal, irradiated, and spleen-shielded rats. Each point is the average of three determinations.

At the end of the first twenty-four hours after injection, when the radioiodine was attached to the *S. typhi* organisms, 68 per cent of the injected activity had been excreted by the normal animals and 47 per cent by the irradiated animals. When the I^{131} was not attached to the vaccine, 88 per cent of the injected activity was excreted by the control animals at the end of the first twenty-four hours and 78 per cent by the irradiated animals. Although there was rapid excretion of I^{131} following injection of I^{131}-labeled typhoid

TABLE IV. AVERAGE ANTIBODY DETERMINATIONS

EXPERIMENT	GROUP	TYPHOID AGGLUTININS DAY AFTER ANTIGEN			HEMOLYSINS DAY AFTER ANTIGEN		
		DAY 0	DAY 4	DAY 6	DAY 0	DAY 4	DAY 6
I	Normal	1:20*†	1:640†	1:2130†	1:30*†	1:30*†	1:240†
	Irradiated	1:20*†	1:20*†	1:67†	1:30*†	1:30*†	1:30†
II	Normal	1:20*†	1:320‡	1:73†	1:30*†	1:90‡	1:800†
	Irradiated	1:20*†	1:20*§	1:20*‡	1:30*†	1:30*§	1:30*†
III	Normal	--	--	1:1710†	--	--	1:7680†
	Irradiated	--	--	1:40†	--	--	1:30*†
	Spleen-shielded	--	--	1:870†	--	--	1:3570†

*Titer less than the number listed.
†The mean of results from three animals.
‡The mean of results from two animals.
§Result from one animal.

vaccine, there was even more rapid excretion when unattached NaI^{131} was given. The difference in percentage excretion in the urine between the control animals and the irradiated animals, although noted in this experiment, was not as marked as in Experiment I. Again, there was no difference in organ distribution to account for this.

Since lead shielding of the spleen has been shown to abolish the inhibitory effect of x-irradiation on antibody production, the third experiment was performed to determine how spleen shielding might affect the metabolism of I^{131}-labeled *S. typhi*.

Experiment III.—The general plan of this experiment was similar to that of Experiment I. One group of animals was subjected to 500 r total body x-irradiation, one group was subjected to 500 r total body x-irradiation with lead shielding of the spleen, and one group was retained as controls. Twenty-four hours after irradiation, 1.5 ml. of I^{131}-labeled typhoid vaccine (about 171,000 counts per minute) and 0.5 ml. of the 0.5 per cent suspension of sheep erythrocytes were given intravenously.

The animals were treated and sacrificed according to the schedule of Experiment I except that no animals were sacrificed fifteen minutes after antigen injection. In addition to the other tissues, the thymus and bone marrow were examined histologically in this experiment.

The results of the determinations of radioactivity in the liver, spleen, lung, urine, and blood are shown in Table III and Fig. 3. The antibody titers are recorded in Table IV.

TABLE V. TOTAL ACTIVITY IN ORGANS COUNTED

EXPERIMENT	GROUP	SUM OF PER CENTS OF INJECTED ACTIVITY IN LIVER, SPLEEN, KIDNEY, LUNG AT 60 MINUTES
I	Normal	79.2*
	Irradiated	80.2
II	Normal	3.5
	Irradiated	3.5
III	Normal	66.3
	Irradiated	70.3
	Spleen-shielded	73.5

*All results are expressed as the mean of results from three animals.

There was no marked difference among the three groups of animals in distribution of radioactivity. The curves of this experiment closely approximate those of Experiment I with one exception. There was a higher percentage of activity in the lungs of animals in all three groups than was found in Experiment I. This was probably due to embolization of small pulmonary vessels since examination of a stained smear of the *S. typhi* antigen preparation showed a moderate degree of clumping of the organisms. This was not true in Experiment I.

The delay in urinary excretion of I^{131} by irradiated animals was not as marked as in the previous experiments.

In summary, it appears that normal, irradiated, and spleen-shielded rats do not differ appreciably in the rate of initial phagocytosis, in the tissue distribution of antigen, or in the rate of antigen breakdown following the single intravenous injection of particulate antigen. Determination of the circulating antibody showed, however, that irradiation depressed antibody formation and that spleen shielding protected the antibody forming mechanism. Histologic material from the tissues of these three groups of animals was examined to determine if there was a difference in the cellular localization of antigen or in the cellular response to antigenic stimulation which might help explain the inhibitory effect of x-irradiation.

HISTOLOGY

Radioautographs.—Experiment I: The spleen showed localization of the labeled antigen in the mantle of pale staining cells surrounding the Malpighian corpuscle and focally in the rest of the red pulp. Thus, all of the antigen appeared to be in the red pulp; the follicles showed no localization (Fig. 4 *A*, *C*). The labeled antigen was distributed focally in the liver and seemed to be within the Kupffer cells. There was no constant relation to any part of the liver lobule (Fig. 4 *B*, *D*). Very little activity was seen in the lungs and kidneys. No activity was found in the lymph nodes.

This pattern was seen in both the normal and irradiated animals.

Experiment II.—There was no activity evident in the sections taken from any animals of this experiment at any time. The same amount of radioactivity had been injected into these animals, and the emulsion of the radioautographs was exposed for six weeks as compared to four weeks in Experiment I.

Experiment III.—The general pattern of distribution of labeled antigen in the normal, irradiated, and spleen-shielded animals was the same as that seen in Experiment I. The lungs, however, showed greater localization of antigen in small arteries, presumably resulting from embolization by clumped organisms.

Goulian[16] studied tissue localization of $CrP^{32}O_4$ after intravenous injection into rabbits, rats, and mice. Distribution in the spleen was in the same areas as observed in the experiments reported here. Distribution in the liver tended to be concentrated in the periphery of the lobules. Preferential localization in the liver was not noted in the experiments reported here. This may have been because a smaller amount of radioactive particles was injected.

As with most radioautographs, it was not possible to determine definitely within which cells the labeled antigen was located. Thus only zones of localization could be determined with certainty.

Stained Sections.—The tissue sections from each experiment were stained with methyl green-pyronin and Azure B. Control sections treated with

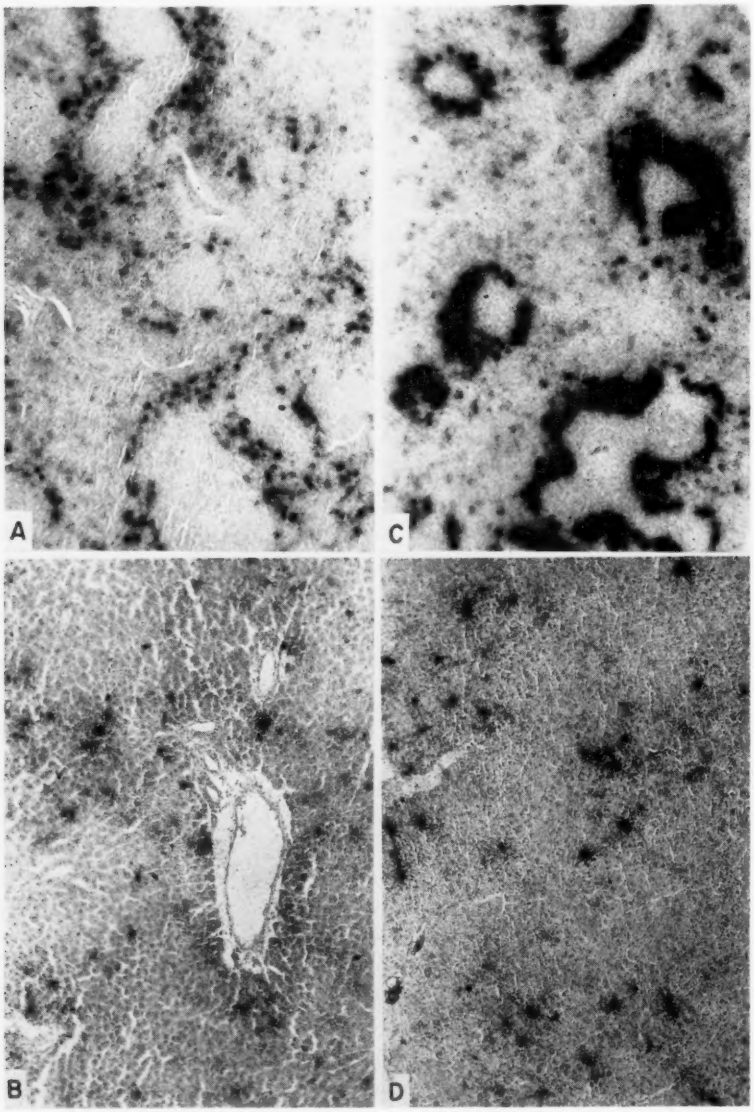

Fig. 4.—Localization of I^{131}-labeled typhoid vaccine in tissues fifteen minutes after intravenous injection. The dark areas are due to reduction of silver particles by beta radiation. Radioautographs counterstained with hematoxylin and eosin. (Magnification *A* and *C* ×40; *B* and *D* ×80.) *A*, Spleen from a normal rat; *B*, liver from a normal rat; *C*, spleen from a rat twenty-four hours after 500 r total body x-irradiation; *D*, liver from a rat twenty-four hours after 500 r total body x-irradiation.

ribonuclease showed loss of pyronin staining and loss of metachromatic Azure B staining. Sections from each animal prepared with each method were studied. The observations were similar. Hence, only the results obtained by the methyl green-pyronin stain will be recorded. Furthermore, it was evident that the pattern of cellular changes and changes in degree of pyroninophilia for corresponding groups in each of the experiments were similar. Therefore,

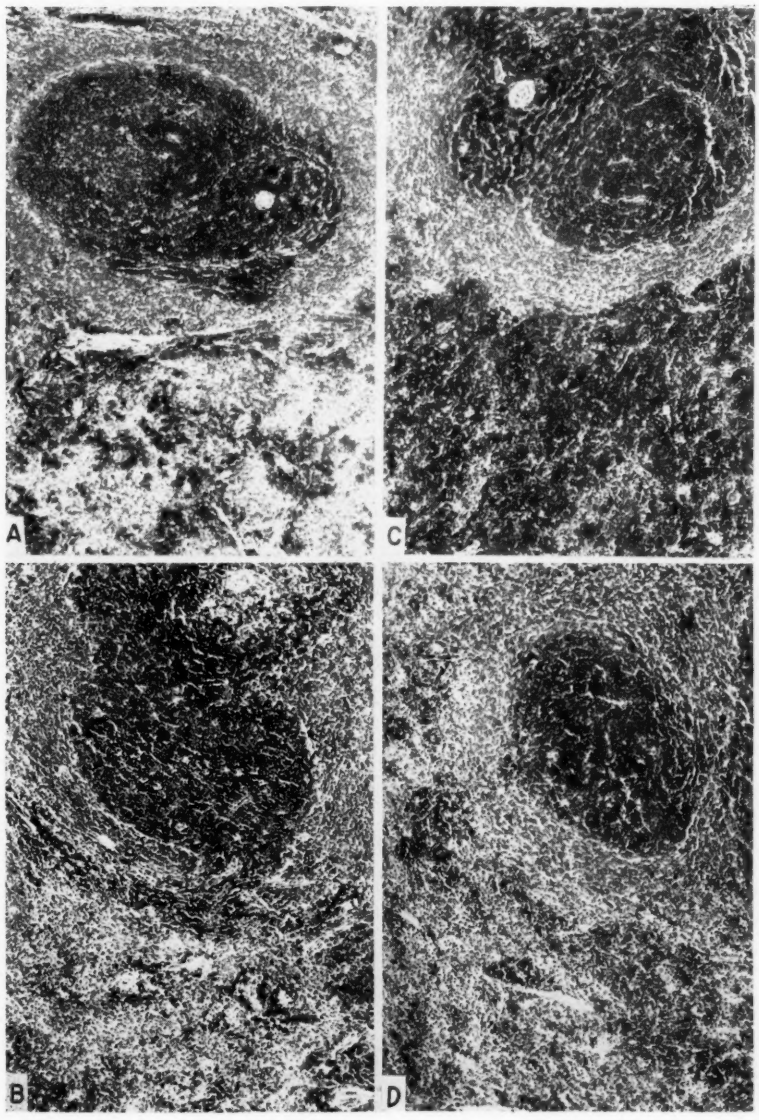

Fig. 5.—Spleens of normal rats after intravenous injection of I^{131}-labeled typhoid vaccine. Methyl green-pyronin stain. (Magnification ×100.) *A*, Fifteen minutes after injection; *B*, one day after injection; *C*, four days after injection; *D*, six days after injection.

the three experiments will be described together. Cells were not named, since rigid criteria have not been established to enable accurate cell identification. Rather, the pattern of cellular reaction will be described.

Spleens of Normal Animals Receiving Antigen.—The histologic pictures in the tissues of the animals sacrificed fifteen and sixty minutes after antigen injection were nearly identical. The Malpighian corpuscles were prominent, and contained in the center of the follicle a group of cells with intensely pyronin-stained cytoplasm (Fig. 5, *A*). These cells made up about 40 to 50 per cent of the area of the corpuscle. Mitoses were fairly numerous. Some nuclear debris was present in the histiocytes of the corpuscle center. Surrounding the Malpighian corpuscle but located in the red pulp there was a fairly wide mantle of rather large cells with light-staining nuclei. About half of these cells showed faint staining of the cytoplasm with pyronin. The rest of the red pulp was only moderately cellular. There were accumulations of small mononuclear cells in the center of which were a few large cells with pyronin-positive cytoplasm. There were also islands of medium-sized cells with small, dark nuclei which were somewhat eccentrically placed. The cytoplasm of these cells stained intensely with pyronin. In the center of these islands there were also a few large cells with cytoplasm which stained less intensely with pyronin. The mantle about the corpuscle and the islands of medium-sized pyronin-positive cells with eccentrically placed nuclei corresponded to the areas showing marked concentration of antigen in the radioautographs.

Changes were apparent in the spleens of animals sacrificed one day after antigen injection (Fig. 5, *B*). The Malpighian corpuscles appeared somewhat larger and were rather uniform in appearance. Large cells with pyronin-positive cytoplasm were still present, but were distributed throughout the corpuscle and not concentrated in the center. The mantle of large cells surrounding the corpuscle was much narrower, and the outer border was less distinct. A greater proportion of the cells in this band stained with pyronin, and the positive cells seemed to stain more intensely. There was some increased cellularity of the red pulp, and the number of cells staining intensely positive with pyronin was slightly increased.

There were marked changes in the spleens of the animals sacrificed on the fourth day after injection of antigen (Fig. 5, *C*). Large cells with pyronin-positive cytoplasm were again present in the centers of the Malpighian corpuscles and made up about 50 per cent of the area of the corpuscle. The corpuscles were increased in size. The mantle of large cells surrounding the Malpighian corpuscle was still narrow but wider than on day one, and the cells stained even more intensely with pyronin. The red pulp showed a striking increase in cellularity. About 50 per cent of the cells of the red pulp were large mononuclear cells with considerable cytoplasm which stained intensely with pyronin. Many of their nuclei were eccentric. The number of mononuclear cells not staining with pyronin was also increased, and the number of large cells which did not stain with pyronin was decreased.

The marked increase in cellularity of the spleen correlated very well with the increase in weight of the spleen observed at four days. The splenic weights expressed in terms of per cent of body weight are recorded in Table VI.

On the sixth day after injection of antigen, the Malpighian corpuscles had much the same appearance as on the fourth day (Fig. 5, *D*). The mantle of large cells around the corpuscle was larger, and there was less staining of the

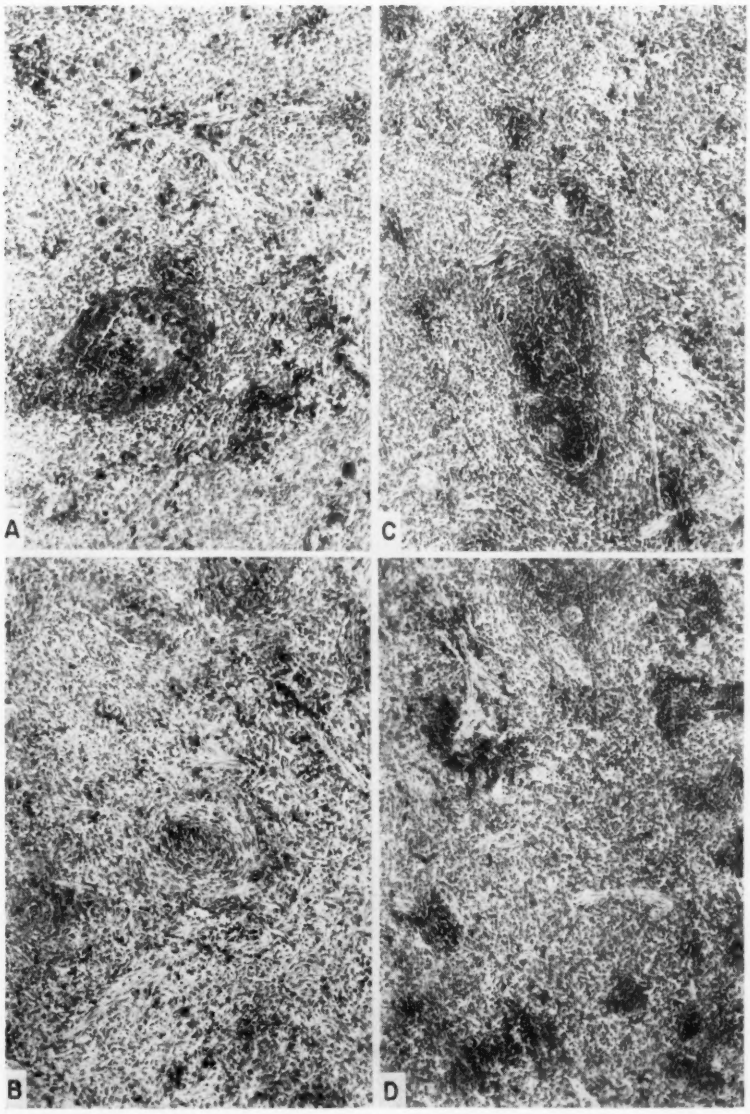

Fig. 6.—Spleens of rats after intravenous injection of I^{131}-labeled typhoid vaccine twenty-four hours after 500 r total body x-irradiation. Methyl green-pyronin stain. (Magnification ×100.) *A*, Fifteen minutes after injection; *B*, one day after injection; *C*, four days after injection; *D*, six days after injection.

TABLE VI. SPLEEN WEIGHT IN TERMS OF PER CENT BODY WEIGHT

EXPERIMENT	GROUP	TIME AFTER ANTIGEN INJECTION			
		60 MINUTES	DAY 1	DAY 4	DAY 6
I	Normal	0.331*	0.355	0.777	--
	Irradiated	0.187	0.178	0.143	--
II	Normal	0.204	0.276†	0.478	0.320
	Irradiated	0.116	0.119	0.111	0.146
III	Normal	0.326	0.398	0.747	0.431
	Irradiated	0.207	0.207	0.180†	0.186
	Spleen-shielded	0.267	0.387	0.636	0.453

*All results are expressed as the mean of results from three animals unless otherwise noted.

†The mean of results from two animals.

cells with pyronin both in terms of intensity and numbers of cells staining. The red pulp was much less cellular than on day four but remained more cellular than on day one. Islands of medium-sized cells, many with eccentric nuclei and intensely pyronin-positive cytoplasm, were larger and more numerous than on day one. Cells which did not stain with pyronin were again prominent.

The decrease in cellularity of the spleen from day four to day six correlated with the decrease in weight of the spleen on the sixth day as shown in Table VI.

Spleens of Irradiated Animals Receiving Antigen.—The histologic pictures in the tissues of the animals sacrificed fifteen and sixty minutes after injection of the antigens were nearly identical. In the spleen, only ghosts of Malpighian corpuscles remained (Fig. 6, *A*). The small mononuclear cells were entirely absent. Many of the large cells with pyronin-positive cytoplasm remained in the region of the center of the corpuscle. Much nuclear debris was present in the phagocytic cells in this area. The wide mantle of cells surrounding the corpuscles which was seen in the normal animals was nearly absent; only a few cells with pyronin-positive cytoplasm remained in this area. The small mononuclear cells were gone from the red pulp. Islands of medium-sized cells with intensely pyronin-positive cytoplasm remained with little apparent change. The vast majority of cells in the red pulp had large, light-staining nuclei and scant cytoplasm which did not stain with pyronin. There was much nuclear debris and some polymorphonuclear leukocyte infiltration in the red pulp. Megakaryocytes normally present in the rat spleen were still present but appeared to be reduced in number.

There were few changes noted in the spleens from animals sacrificed on the day following antigen injection (Fig. 6, *B*). There was less nuclear debris in the phagocytic cells, and the polymorphonuclear infiltration was decreased.

On the fourth day after antigen injection, the islands of intensely pyroninophilic cells remained stationary. It was at this time that normal animals showed the greatest degree of hyperplasia and increased pyronin-staining of the red pulp. The nuclear debris had entirely disappeared from the spleen. There was a slight increase in the number of large cells with pyronin-positive cytoplasm in the region of the Malpighian corpuscles (Fig. 6, *C*). The red pulp

was still largely composed of large cells with light-staining nuclei and scant cytoplasm which did not stain with pyronin. The megakaryocytes were less numerous.

On the sixth day after antigen injection there were a few small mononuclear cells present again in the region of the Malpighian corpuscles (Fig. 6, *D*). There was also a slight increase in the number of cells with pyronin-

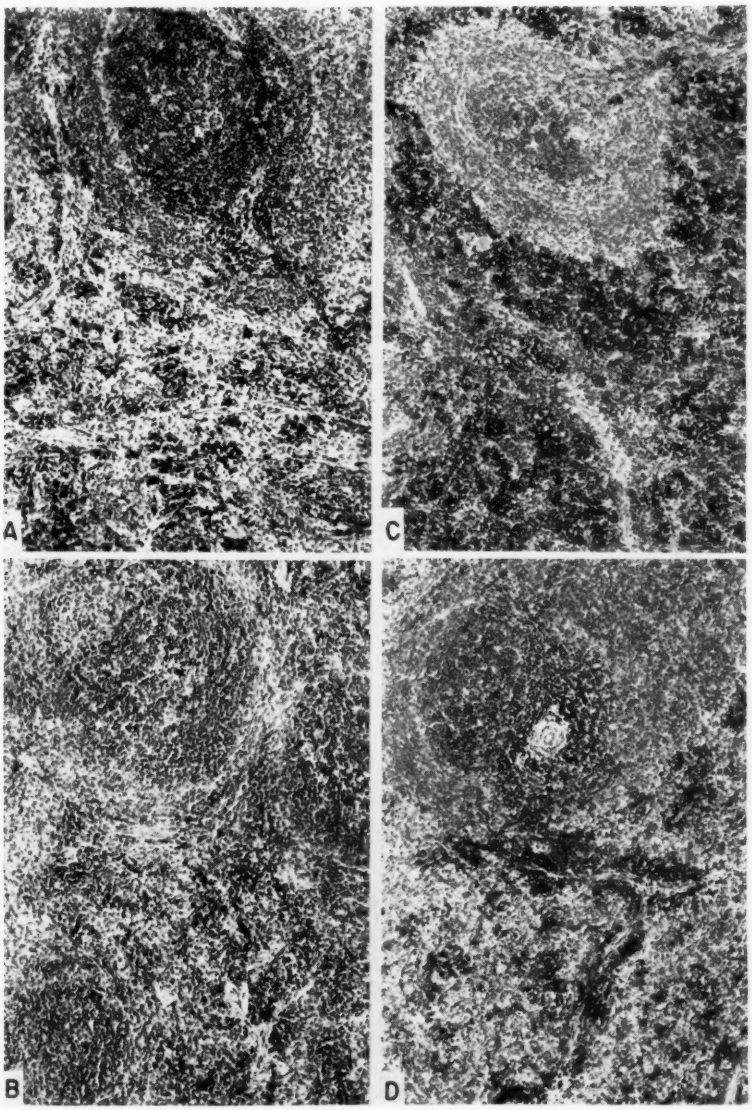

Fig. 7.—Spleens of rats after intravenous injection of I^{131}–labeled typhoid vaccine twenty-four hours after 500 r total body x-irradiation with lead shielding of the spleen. Methyl green-pyronin stain. (Magnification ×100.) *A*, Fifteen minutes after injection; *B*, one day after injection; *C*, four days after injection; *D*, six days after injection.

positive cytoplasm in the mantle surrounding the corpuscle. The red pulp as a whole was still relatively cell poor. There was much more hemosiderin present than in the normal animals.

Spleens of Spleen-Shielded Animals Receiving Antigen.—At each of the intervals the spleens from antigen injected, spleen-shielded animals showed changes similar to the normal animals which had received antigen (Fig. 7, *A, B, C,* and *D*). However, in all cases, there was a decrease in the number of small mononuclear cells in the Malpighian corpuscles and in the red pulp. Hence, the corpuscles were smaller. The pattern of cellular proliferation and the changes in pyroninophilia were similar to those described in normal animals.

Spleens From Noninjected Animals.—The spleens from the noninjected animals in each group showed the same appearance as that described for the injected animals fifteen or sixty minutes after injection.

Other Organs From Normal Animals Receiving Antigen.—At no time after injection were there appreciable changes in cell proliferation or pyronin staining of the liver, lymph node, thymus, bone marrow, kidney, or lung of these animals.

Other Organs From Irradiated Animals Receiving Antigen.—The lymph node, thymus, and bone marrow showed the changes described by others following irradiation.[17] No changes in reaction to the antigen were observed. The liver, kidney, and lung showed no change.

Other Organs From Spleen-Shielded Animals Receiving Antigen.—The lymph node, thymus, and bone marrow showed the changes described by others in spleen-shielded animals.[2, 18] No changes relative to antigen injection were noted. Especially noteworthy was the prompt regeneration of the lymph node and bone marrow as compared with the irradiated animals without spleen-shielding. The liver, kidney, and lung showed no change.

DISCUSSION

Before discussing the results and their significance, it is important to determine the validity of the labeling method employed in this study.

The method of attaching radioiodine to *Salmonella typhi* organisms as described gave a constant degree of antigen iodination. The organisms retained most of their original antigenic make-up. They agglutinated as readily as noniodinated organisms in rabbit sera prepared against normal *S. typhi,* and injection of the labeled organisms into normal rats resulted in sera which showed a high titer against noniodinated *S. typhi.*

Warren and Dixon[19] and Fine and Seligman[20] have demonstrated in vitro the stability of I^{131}-labeled proteins. Dixon and associates[21] have shown that protein antigens seem to retain the I^{131} label in vivo as long as they are immunologically active. With particulate antigen, another factor must be considered. The labeled proteins must not become detached from the particle except by the metabolic process with which the animal disposes of them. In vitro studies of the labeled typhoid vaccine showed only slight losses of radioactivity by the organisms after seven days incubation in rat serum.

It appears that injection of I^{131}-labeled antigen into an animal which has been pretreated with normal iodine results in a level of activity in the tissues which represents iodine still bound to the antigen. Urinary excretion of radioactivity represents the amount of antigen that has been digested. The iodine is attached to the tyrosine groups of the antigen protein. As the antigen is broken down, iodide, diiodotyrosin, and peptide chains containing diiodotyrosine are formed.[22] These substances are not appreciably incorporated either by synthesis or interchange into the body proteins.[21] Administration of large amounts of iodine "saturates" the thyroid and prevents uptake of I^{131}; I^{131} administered to a "saturated" animal is largely excreted in the urine.[23]

The results of this study emphasize the role of the spleen in antibody formation in the rat following a single injection of particulate antigen intravenously.[3] They show that a large proportion of the antigen is localized in the spleen and that some of it is detectable until antibody formation is evident. The histologic reaction which occurs is limited to the spleen and is found in only that part of the spleen where antigen is localized. It occurs at the time when antibody is presumably being formed and subsides as the circulating antibody reaches its peak concentration.

The inhibitory effect of total body x-irradiation on antibody production has been known for many years. However, the mechanism of irradiation inhibition is still obscure. Clemmesen[24] and Andersen[25] have suggested that phagocytosis is inhibited by irradiation but gave no evidence to support this hypothesis. Studies of Chrom[26] suggest impairment of the phagocytic ability of the reticuloendothelial system in mice following irradiation. However, Barrow and co-workers[27] showed that x-irradiation did not depress phagocytosis of intravenously administered colloidal $gold^{198}$ in the rabbit. The studies reported here also demonstrated that phagocytosis is not altered by 500 r total body x-irradiation in the rat.

Taliaferro and collaborators[28] suggest that inhibition of antibody formation by irradiation may be due to prevention of localization of antigen in antibody forming sites and/or derangement of the early metabolism of the antigen. The studies reported here have shown no alteration of localization (either gross or microscopic) or metabolism of I^{131}-labeled typhoid vaccine given twenty-four hours after 500 r total body x-irradiation in the rat. It is possible, however, that the mechanisms suggested by Taliaferro operate intracellularly at a level not detectable by the methods used in this study.

There was an early association of depression of antibody production by x-irradiation with damage to the lymphoid and myeloid elements.[29] The histologic reactions following antigenic stimulation have been known for many years and have been re-emphasized recently.[30-34] However, there are no published reports of the histologic reaction following antigenic stimulation in irradiated animals.

The studies reported here show hyperplasia of the splenic red pulp and increase in pyronin staining of the cells *in the areas of antigen localization*

occurring in association with antibody production. When x-irradiation inhibited antibody formation, this reaction did not occur in the spleen. Pyronin selectively stains ribonucleic acid when used with ribonuclease control.[35] Ribonucleic acid levels in tissues and cells correlate with the rate of protein synthesis.[36-39] It appears, therefore, that following antigenic stimulation, certain cells of the splenic red pulp in normal and spleen-shielded animals are engaged in protein synthesis. This evidence of protein synthesis was not present in the irradiated animals.

The inhibitory effect of total body x-irradiation in the rat following intravenous injection of particulate antigen appears to be related to alteration of cellular response in the spleen rather than to alteration of antigen metabolism.

SUMMARY

Adult male rats were given 500 r total body x-irradiation with and without lead shielding of the spleen. Twenty-four hours later these rats and normal controls were given a single injection of I^{131}-labeled typhoid vaccine intravenously. Blood stream clearance, tissue distribution, and excretion of I^{131} were studied. The histologic reaction to antigenic stimulation was followed at intervals of one, four, and six days after antigen injection.

There was no difference in rate of phagocytosis, tissue localization, or rate of disappearance of antigen from the tissues in the three groups of animals.

Radioautographs showed localization of antigen in the red pulp of the spleen, in the mantle of cells around the Malpighian corpuscles and focally in the pulp.

The histologic reaction of the spleen following antigenic stimulation in normal and spleen-shielded animals consisted of hyperplasia of the red pulp and increase in pyronin staining of the cells. This pattern was not observed in irradiated animals.

The significance of these findings with regard to the mechanism of irradiation damage to antibody formation is discussed.

The authors wish to express appreciation for the encouragement and advice of Dr. David Talmage, Dr. L. O. Jacobson, and Dr. Paul R. Cannon.

The photomicrographs were made by Jean M. Crunelle of the Photography Department of Billings Hospital of the University of Chicago.

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RENAL EXCRETION OF CREATININE AND INULIN IN MAN

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EVER since 1937, when one of the authors (E. E. M.) together with Popper recommended the endogenous creatinine clearance as a clinical measure of the glomerular filtration rate,[1] a controversy over the physiologic significance of this clearance has persisted. Apart from conflicting results based on determinations of total creatinine chromogen in blood by different analytical methods[2-17] which were largely summarized by Smith,[18] there is no complete accord on the relation of the clearance of "true" creatinine to the glomerular filtration rate as estimated by means of inulin. While the average creatinine/inulin clearance ratio in persons without renal disease has been reported by different investigators to be close to unity,[12, 18-21] the range of these clearance ratios has often been considerable: 0.8 to 1.5 was found by Miller and Winkler with the use of their enzymatic method of analyzing creatinine,[19] 0.82 to 1.26 was the range encountered by Hare and his associates,[20] and closely similar extreme values were noted by Mattar and co-workers,[21] all of whom employed the adsorption-elution (Lloyd's reagent) method.[22] Brod and Kotatko, using the same analytical principle, found the clearance ratio in all but one of the normal subjects which they studied to be 1.0.[12] However, Taggart reported the endogenous creatinine clearance, as determined by Miller's and Winkler's technique, to exceed the "filtration rate" by an average of 25 per cent.[23] A clearance ratio of well above 1.0 has generally been noted in patients with renal disease,[12, 18-21, 24] while in congestive heart failure, ratios distinctly below unity have been encountered.[24]

The inference through all these reports is that the inulin clearance represents the glomerular filtration rate. This assumption has recently been challenged by Ferguson and his associates who studied the influence of variations in inulin plasma concentration upon the renal excretory rate of inulin and concluded that up to 15 per cent of inulin filtered through the glomeruli may normally be subject to tubular reabsorption.[25] This finding, if verified, clearly would not only call for a re-evaluation of published results on the creatinine clearance but it would also tend to invalidate a host of data accumulated in the fields of renal physiology and pathology during the past fifteen years.[18]

The experiments to be reported were undertaken in an attempt to clarify the two problems specified above and therefore involved, in principle, (a) a comparison of clearances of endogenous creatinine and creatinine chromogen with those of inulin, and (b) a re-examination of the relation of the inulin clearance to the inulin plasma level, supported by the simultaneously determined creatinine clearance.

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MATERIAL AND METHODS

The clearances of inulin (C_{IN}) and creatinine (C_{CR}) were determined concomitantly in 13 subjects with no apparent disturbance of renal function (hereafter called "normals") and in 21 patients suffering from either a primary renal disorder or from renal involvement secondary to cardiovascular disease, infection, or intoxication (Table I). Criteria for the absence of renal disease included normal urinary findings concerning protein, specific gravity and microscopic sediment, blood pressure readings within normal limits, and plasma creatinine (P_{CR}) and urea nitrogen levels below 1.2 (1.0 in females) and 15 mg. per 100 ml., respectively. In some experiments, creatinine chromogen (C_{CHR}), p-aminohippurate (C_{PAH}) and uric acid (C_{UA}) clearances also were measured, as well as the extraction ratios for creatinine and inulin, according to the technique of Warren and associates.[26] In addition, clearances were determined before and after administration of creatinine and of Benemid by mouth and in association with changes in the rate of inulin infusion, as described by Ferguson and coworkers.[25]

The clearance techniques of Smith were employed.[18] After a priming intravenous injection containing between 20 and 50 ml. of a 10 per cent inulin solution (and, in some cases, between 1 and 2.5 ml. of a 20 per cent p-aminohippurate [PAH] solution), the sustaining infusion was started, containing about 2 to 3 times the amounts of inulin (and PAH) diluted with saline. The volumes were adjusted to the patient's size and degree of renal insufficiency, if present. Constant delivery of the infused fluid was assured by the use of a Bowman pump. Changes in the inulin plasma concentration were readily accomplished by either temporarily discontinuing the infusion or adding another priming injection, followed by decreased or increased pump delivery speed, as desired. In every instance, a minimum of forty minutes of constant rate of infusion was allowed for equilibration before clearance measurements were begun. A waiting period of one hour was observed after creatinine or Benemid administration. Urine was obtained through indwelling multi-eyed catheters, each collection combined with 2 bladder washings using 10 to 20 ml. of distilled water and equal amounts of air, followed by manual expression of the bladder whenever feasible. Venous blood was usually withdrawn both at the beginning and end of the first collection period and, thereafter, toward the end of every subsequent period; the plasma values used in calculating clearances were derived from plotting the known concentrations semilogarithmically against time and extrapolating back 2.5 minutes from the middle of each period. Occasionally, only midtime blood collections were made and used directly for clearance calculations (marked ‖ in Table I). The duration of clearance periods usually ranged from ten to fifteen minutes, with rare extensions up to twenty minutes because of low urine flow or technical difficulties.

Inulin was determined in heparinized plasma and in urine by the resorcinol method of Higashi and Peters,[27] PAH by the method of Smith and collaborators.[28] Plasma and urine samples collected just prior to the start of the infusion served as colorimetric blanks (readings were usually not below 96 per cent transmittance). Creatinine was measured by Hare's adsorption-elution method,[22] using for deproteinization either 20 per cent trichloracetic acid or a combination of 5 per cent tungstate with $\frac{2}{3}$ N sulfuric acid.[29] Creatinine chromogen was estimated either in "neutral" tungstate filtrates (in early experiments—marked ‡ in Table I—when their incomplete creatinine yield had not been recognized) or in the same acid tungstate filtrates as creatinine.[29] Uric acid was measured in accordance with procedures by Buchanan and associates[30]* and by Brown.[31] All chemical determinations were performed on duplicate samples and their accuracy repeatedly checked by means of recovery experiments. At the Communicable Disease Center (C. D. C.) Laboratory, a Beckman model DU spectrophotometer was utilized for the creatinine and chromogen analyses and an Evelyn photoelectric colorimeter for the other procedures; at the Veterans Administration (V. A.) Laboratory, creatinine and inulin measurements on aliquots of specimens from 4 cases (Table I) were carried out by means of a Coleman Universal Model Spectrophotometer.

*The direct photometric procedure was adopted without the use of uricase (Symposium on Azotemia of American Society of Clinical Pathologists, Chicago, Oct. 16, 1951).

RESULTS

Comparison of C_{CR} and C_{CHR} with C_{IN}: Among the "normal" subjects, the mean C_{CR}:C_{IN} ratio was only slightly above 1.0, while the range of extremes and the differences between subjects were considerable (Tables I and II). From the regression line and the width of the 95 per cent confidence band in Fig. 1,

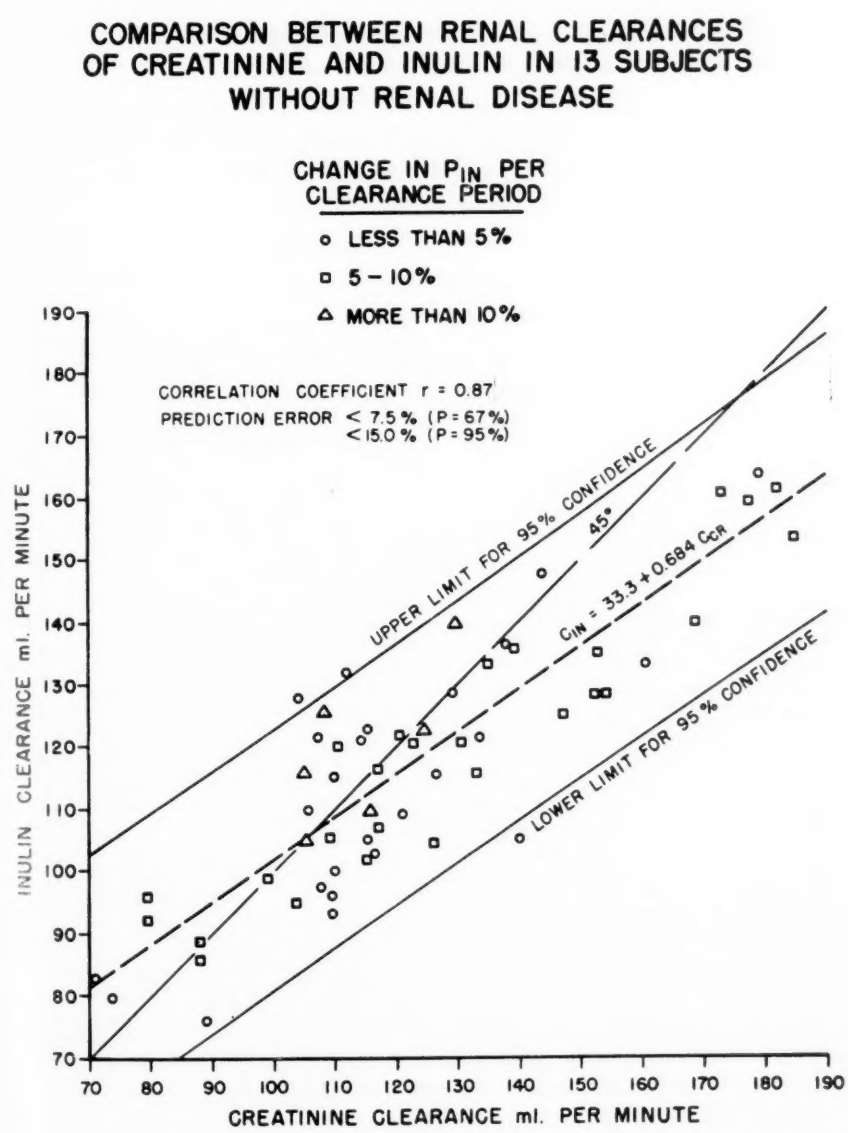

Fig. 1.—Individual clearance periods are indicated by circles, squares or triangles, the different symbols corresponding to different degreesof change in plasma inulin concentration (P_{IN}) during each period. 45° marks the line of equal clearances ($C_{IN} = C_{CR}$). The regression line for all the clearances plotted is indicated by the formula allowing prediction of C_{IN} from C_{CR}. *P* denotes probability

TABLE I. SIMULTANEOUS CLEARANCES (C) OF INULIN (IN), CREATININE (CR), CREATININE CHROMOGEN (CHR), SODIUM P-AMINOHIPPURATE (PAH) AND URIC ACID (UA).

SUBJECT SEX AND AGE	DIAGNOSIS	PERIODS*	PLASMA (MG. PER 100 ML.) IN	CR	CHR	CLEARANCES* (ML. PER MIN.) IN	CR	CHR	C_{CR}/C_{IN}	C_{CHR}/C_{IN}	C_{PAH}/C_{IN}	REMARKS†
					A. Subjects Without Renal Disease							
E. W.—F 30	Pneumonia (conv.)	2	33.4	0.65	0.94	116.1	130.4	97.7	1.12	0.34	5.47	
J. D.—F 29	Pneumonia (conv.)	2	30.2	0.87		130.4	127.0		0.98			V. A.
L. S.—F 37	Multiple sclerosis	3	51.2	0.62		90.3	76.8		0.84			
B. S.—F 28	Hodgkin's disease	2	56.4	0.63		109.8	107.6		0.98			
A. J.—F 32	Pneumonia (conv.)	2	40.4	0.44	0.68‡	132.4	133.4	89.1	1.01	0.68		
B. A.—F 27	Lymphogranuloma	2	52.2	0.44	0.74‡	162.8	180.6	114.9	1.11	0.71		
L. L.—F 33	Varicose veins	2	64.0	0.70	0.98‡	118.8	125.4	96.0	1.06	0.81		V. A.
A. B.—F 47	Syphilis	2	59.1	0.66		112.9	116.0		1.03			
G. P.—F 23	Peptic ulcer	2	38.0	0.70		102.2	109.7		1.08			V. A.
M. C.—F 19	Subarachnoid hemorrhage (conv.)	3	24.0	0.66		125.2	128.6		1.02		6.81	
W. R.—M 25	Mumps	3	20.2	0.84		100.7	108.0		1.06		5.72	
G. E.—M 59	Pleural effusion	3	24.0	0.70	0.92	122.1	111.0	91.2	0.90	0.74		← —C 3
		2	21.1	3.26	3.47	124.2	285.5	268.1	1.74	1.70		
J. J.—M 30	Neurosis	3	25.6	1.00		108.2	133.1		1.23§			0.11 ←B
		3	27.2	1.00		100.3	119.8		1.18			0.47 ←C 5
	(Next day)	3	22.6	6.46		117.6	135.6		1.15§			0.12 ←B
		4	20.0	5.64		120.6	128.2		1.06			0.44
					B. Subjects With Renal Disease							
L. A.—M 24	Acute glomerular nephritis (conv.)	3	21.6	1.01		83.4	88.5		1.06		5.76	
A. G.—M 13	Acute glomerular nephritis (conv.)	3	20.2	0.79		93.0	97.3		1.04		5.30	
W. O.—M 14	Acute glomerular nephritis (conv.)	3	17.4	1.27	1.48	55.1	80.8	75.5	1.46	1.37	17.1	
W. S.—M 19	Subacute glomerular nephritis	4	46.8	3.10	3.07‡	19.5	27.1	26.6	1.40	1.36	10.74¶	
M. B.—F 32	Chr. glomerular nephritis	3	30.2	6.33	6.46	4.2	5.6	5.6	1.38	1.35	3.51¶	

*Number of consecutive clearances determined. Corresponding values in other columns represent averages. Clearances measured after deliberate changes in inulin infusion rate (and equilibration) are not included in this Table but are represented in Tables II, III and V and in Figs. 1, 2, 3 and 4.

†Fractions under REMARKS represent $\frac{C_{UA}}{C_{IN}}$ ratios.

‡Indicates use of "neutral" tungstate filtrates.

§The apparent lowering of the mean ratio following Benemid is not significant because of considerable overlapping of individual ratios.

‖Indicates midpoint blood collections (instead of the usual blood collections at the beginning and end of periods).

¶Denotes plasma PAH levels above 3.0 mg. per 100 ml.; in all other instances, levels ranged between 0.5 and 3.0 mg. per 100 ml.

(—Denotes ingestion of 1 Gm. of Benemid.

V.A.—Refers to plasma and urine specimens, aliquots of which were analyzed in the V.A. laboratory.

C—Denotes ingestion of creatinine; the adjoining number indicates the amount in grams.

Conv.—convalescent.

4H—Means 4 hours.

4D—Means 4 days.

4H "after eating"

V.A.—Refers to plasma and urine specimens, aliquots of which were analyzed in the V.A. laboratory.
C—Denotes ingestion of creatinine; the adjoining number indicates the amount in grams.
Conv.—convalescent.
4H—Means 4 hours.
4D—Means 4 days.
4 s—Means "one sitting."

O. P.—F 42	Nephrosis	3	33.7	0.77		67.5	94.6		1.40		7.88	0.32
												←B
		3	28.6	0.79		61.4	94.6		1.53		7.94	0.50
W. M.—M 63	Chronic pyelonephritis	3	43.8	1.41		24.5	30.0		1.22			0.46
												←B
		2	54.0	1.40		24.8	29.7		1.20			0.54
N. C.—M 53	Chronic pyelonephritis	3	39.2	2.03		26.3	35.9		1.36			0.51
												←B
		3	46.4	1.95		30.0	35.2		1.30			1.02
E. Z.—F 25	Chronic pyelonephritis	2	58.8	10.86	11.68‡	2.8	4.8	4.9	1.70	1.74	10.36	
M. R.—F 26	Hypertension	2	38.2	0.73	0.82	50.8	58.6	52.9	1.15	1.04	5.56	
S. G.—F 53	Gastric sarcoma; hypertension	6	35.8‖	1.02		72.0	89.0		1.23		5.08	
W. T.—F 31	Cushing's syndrome; hypertension	3	27.3	0.91	1.12	118.3	136.6	117.2	1.16	0.99		V. A.
												←C 8
		3	24.8	10.61	9.98	111.7	175.6	195.8	1.58	1.76		
M. C.—F 59	Hypertension; diabetes	3	50.4	2.26	2.88	19.6	23.3	19.4	1.18	0.99	6.55¶	
H. A.—F 38	Diabetes; chronic pyelonephritis	3	39.2	1.22		41.4	46.8		1.13		5.44	
												←H. S.
	postural hypotension	2	43.4	1.20		33.8	40.2		1.18		5.55	
												←S.
		2	45.6	1.16		24.5	29.6		1.20		5.56	
P. J.—M 40	Malignant hypertension	4	21.8	3.18	3.50	18.3	24.3	22.4	1.32	1.22	3.19	
R. C.—M 36	Malignant hypertension	3	42.8	9.23	9.04‡	7.3	8.8	9.6	1.20	1.30		
A. R.—M 54	Malignant hypertension	2	55.8‖	11.36		1.4	1.4		1.08		3.44¶	
A. P.—F 36	Malignant hypertension	2	32.9	15.37	17.26	0.7	1.0	1.0	1.76	1.72	4.76¶	
												←4 H
		2	28.2	15.38	17.22	0.7	1.2	1.2	1.76	1.72	8.21	
A. H.—M 33	Chr. lead poisoning	2	47.6	1.69		82.5	91.2		1.10		7.06¶	
												←4 D
		3	44.8	1.60		94.0	105.8		1.13		5.94	
O. J.—M 40	Chr. congestive failure	3	23.9	0.92		66.0	53.1		0.80		1.59	
O. H.—M 65	Peritonitis	3	34.2	2.24		34.3	31.7		0.92			

TABLE II.

A. SUMMARY OF CLEARANCE RATIOS

CLEARANCE RATIOS	NO. OF SUBJECTS	NO. OF PERIODS	EXTREMES OF RATIOS	STANDARD DEVIATIONS¶ ALL RATIOS	BETWEEN SUBJECTS	WITHIN SUBJECTS	MEAN RATIOS
$C_{CR}:C_{IN}$	13	58	0.81-1.33	0.12	0.23	0.06	1.04
	21	*93*	*0.72-1.83*	*(0.22)*	*(0.45)*	*0.07*	*1.25*
Miller*	*9*	*28*	*0.83-1.71*	*(0.30)*	*(0.53)*	*0.11*	*1.31*
C.D.C.†	4	15	0.93-1.20	0.09	0.18	0.04	1.09
V.A.†	4	15	0.90-1.27	0.12	0.19	0.09	1.13
$C_{CHR}:C_{IN}$	5	20	0.65-0.86	0.07	0.14	0.02	0.76
	10	*39*	*0.90-1.86*	*(0.24)*	*(0.47)*	*0.07*	*1.29*
$C_{CHR}:C_{CR}$	5	20	0.58-0.86	0.08	0.16	0.03	0.71
	10	*39*	*0.79-1.11*	*0.08*	*0.14*	*0.04*	*0.94*

B. ANALYSIS OF VARIANCE OF $C_{CR}:C_{IN}$ RATIOS FROM TWO LABORATORIES†

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES‖	ESTIMATES OF VARIANCE	F-RATIO
Laboratories	1	0.0154	0.0154	3.6‡
Subjects	3	0.2001	0.0667	15.5§
Interaction (LxS)	3	0.0051	0.0017‡	
	>25	>0.1063	>0.0043	
Error	22	0.1012	0.0046	

Figures in italics refer to patients with renal disease.

In those subjects whose plasma inulin level was deliberately varied in the course of the experiment (Table V, Figs. 3 and 4), clearance periods before and after the change in infusion rate were utilized in this and the following Table.

*Data of Miller and associates.[24]

†Results of analyses performed at C.D.C. and V.A. laboratories on aliquots of the same specimens from 4 subjects (3 "normal," 1 "renal") listed in Table I.

( ) Denotes lack of statistical validity due to heterogeneity of the group analyzed.

‡Denotes lack of significance at the 0.05 probability level.

§Denotes significance at the 0.01 probability level.

‖Of deviations from mean ratio.

¶Standard deviation $= \sqrt{\text{estimated variance}}$

TABLE III. VARIATION OF SUCCESSIVE CLEARANCE MEASUREMENTS

A. ANALYSIS OF VARIANCE

SOURCE OF VARIATION	DEGREES OF FREEDOM	ESTIMATES OF VARIANCE C_{IN}	ESTIMATES OF VARIANCE C_{CR}	F-RATIOS C_{IN}	F-RATIOS C_{CR}
Pairs of clearances	24	806.6	1,334.3	7.7§	12.0§
	29	*1,999.5*	*2,647.9*	*165.9§*	*126.8§*
1st period vs. 2nd period	1	393.1	47.4	3.8‡	0.4‡
	1	*0.9*	*5.8*	*0.1‡*	*0.2‡*
Error	24	104.3	110.9		
	29	*12.1*	*24.3*		

B. DERIVATION OF COEFFICIENTS OF VARIATION

CLEARANCES	MEANS OF CLEARANCES 1ST PERIOD	2ND PERIOD	1ST AND 2ND PERIOD COMBINED	S.D.*	C.V.†
IN	119.6	113.9	116.8	10.22	8.7
	42.1	*41.9*	*42.0*	*3.47*	*8.3*
CR	123.4	121.5	122.5	10.53	8.6
	49.4	*48.7*	*49.0*	*4.93*	*10.1*

For explanation of figures in italics, see Table II.

*Standard deviation $= \sqrt{\text{Error variance}}$

†Coefficient of variation $= \frac{100 \text{ S.D.}}{\text{Combined means}}$

‡ and §See Table II.

it is evident that the error in predicting C_{IN} from C_{CR} can be quite large. This error (based on the variance from regression [Fig. 1]) may be expected to be numerically less than 7.5 per cent of the actual C_{IN} value about two-thirds of the time and practically never to exceed 15 per cent. C_{CHR} values were, on the average, about 24 per cent lower than corresponding C_{IN} values; their number was too small for calculation of valid regression data.

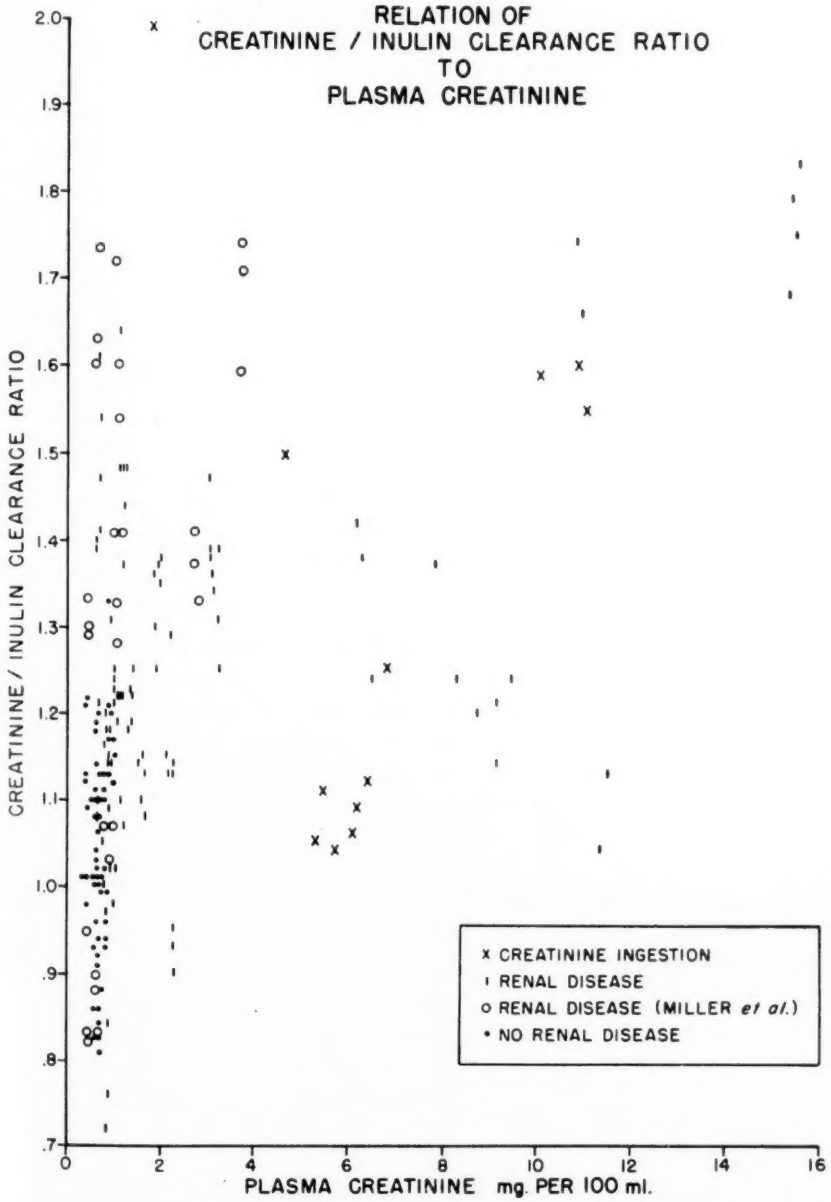

Fig. 2.—Each symbol corresponds to one clearance period.

In the group of 21 patients with renal disease, the mean C_{CR} and C_{CHR} values were 25 and 29 per cent, respectively, greater than the corresponding C_{IN} values. The lack of homogeneity of the group appears to express itself in the wide range of extremes of clearance ratios and would invalidate any inferences based on such quantities as mean and standard deviation. Only the C_{CHR}:C_{CR} ratios did not show a wider range than in the "normal" subjects, but the mean ratio was now 0.94 as against 0.71. Actually, with the exception of 7 periods in 4 cases (M. B., E. Z., R. C. and A. P., Table I) manifesting marked azotemia, these ratios were always below 1.0 though to a far lesser degree than in the "normals," a difference which is presumably due to suppression of the noncreatinine chromogen fraction in the presence of increased creatinine concentrations in serum.[29, 32] The C_{CR}:C_{IN} ratio was distinctly below 1.0 in the 2 cases of "extrarenal" azotemia studied: One (O. H.), suffering from perityphlitic abscess and peritonitis, had a plasma urea nitrogen of 120 and a creatinine of 2.25 mg. per 100 ml., the other (O. J.), a case of chronic congestive failure, had a slight elevation of the urea nitrogen (17.6 mg.) and a normal creatinine (the urine containing protein and granular casts). The average C_{CR}:C_{IN} ratio in the renal group with exclusion of these 2 cases was 1.29 (range 0.98 to 1.83). Of these 19 cases, only 8 had ratios clearly above the normal range, i.e., above 1.33. Their P_{CR} ranged from normal (0.77 mg. per 100 ml., O.P.) to excessively high levels (15.4, A.P.). These considerations and a study of Fig. 2, which includes pertinent findings by Miller and his associates,[24] indicate that the C_{CR}:C_{IN} ratio is not a direct function of the P_{CR} level or of the degree of uremia.

Influence of Fluctuations of P_{IN} upon C_{CR}:C_{IN} ratio: Since unavoidable fluctuations in P_{IN} often encountered in spite of a constant rate of inulin infusion (P_{CR} being practically constant*) conceivably could have an effect on the C_{CR}:C_{IN} ratios, the percentage differences of successive P_{IN} determinations among the "normals" were calculated and compared with the corresponding ratios (Fig. 1). Analysis of covariance revealed no significant differences in the slopes of regression lines, whether the change in P_{IN} was 5 per cent or less (25 periods), between 5 and 10 per cent (27 periods), or even greater than 10 per cent (6 periods).

Variation of Successive Clearance Measurements: An attempt was made to determine whether the systematic decrease which has been found by Davies and Shock[33] among successive C_{IN} measurements also prevailed in the present study and perhaps contributed to the observed differences between C_{IN} and C_{CR}. Analysis of 25 pairs of clearance periods among the "normals" and of 30 pairs in the renal group (Table III) did show slight average decreases for C_{IN} and C_{CR} which, however, were not statistically significant. Coefficients of variation calculated indicate that the inulin clearances of 2 successive periods can be

*Actually, there was a slight downward trend in P_{CR} throughout many of the clearance experiments, the differences between individual P_{CR} values being significantly greater than the experimental error. We can offer no reasonable explanation for this phenomenon. It can hardly be attributed to plasma dilution by the inulin infusion usually given at a rate of less than 1 ml. per minute.

expected to fall within about 8.5 per cent in two out of three instances and hardly ever beyond 17 per cent. The coefficients for creatinine clearance were of the same order of magnitude.

Influence of Analytical Error on C_{CR}:C_{IN} Ratios: Aliquots of plasma and urine of 4 cases were analyzed simultaneously in 2 different laboratories (C.D.C. and V.A.) and by different technicians, each using her own reagents and equipment (Table II). Small differences between corresponding analytical results caused the V.A. C_{CR}:C_{IN} ratios to be, on the average, somewhat higher than the C.D.C. ratios. This difference, however, lacked statistical significance and both laboratories furnished C_{CR}:C_{IN} ratios above unity for most of the test periods in these cases. Consequently, analytical errors could not play a major role in the relation between C_{CR} and C_{IN}.

Comparison of Renal Extraction Ratios for Creatinine and Inulin: Catheterization of the right renal vein in 2 "non-renal" cases resulted in extraction ratios for inulin which were higher than those for creatinine (Table IV). These findings would suggest C_{CR}:C_{IN} ratios below 1 in these cases, but, unfortunately, neither simultaneous nor subsequent urine collections and clearances could be obtained.

TABLE IV. COMPARISON OF RENAL EXTRACTION RATIOS (E) FOR CREATININE (CR) AND INULIN (IN)

PATIENT AND DIAGNOSIS	SUCCESSIVE SAMPLINGS*	PLASMA				EXTRACTION RATIOS			
		FEMORAL ARTERY		RT. RENAL VEIN					
		CR	IN	CR	IN	CR	IN	MEAN CR/IN	PAH
		MG. PER 100 ML.†				PER CENT			
J. H.	1	0.60	26.7	0.51	22.5	15.1	15.7		28.5‡
Lung abscess	2	0.57	26.3	0.48	21.5	15.3	18.2	0.91	26.7‡
	3	0.56	26.3	0.46	21.5	16.7	18.2		28.7‡
W. H.	1	0.68	42.3	0.62	36.7	8.9	13.3		93.2
Convalescence	2	0.65	40.4	0.59	35.0	9.3	13.4	0.74	92.5
from acute alcoholism	3	0.59	40.1	0.53	35.6	9.7	11.2		92.8

*10 ml. of blood were withdrawn simultaneously by two persons from the femoral artery and the cubital end of a catheter whose tip had, under fluoroscopic control, been placed in the right renal vein. Intervals between samplings were about five minutes.
†Results are means of duplicate analyses of duplicate protein-free filtrates.
‡Incorrect values due to inadvertent sulfonamide medication.

Exogenous Creatinine Clearance: Creatinine ingestion in the course of routine clearance experiments resulted in sharp increases of the C_{CR}:C_{IN} and C_{CHR}:C_{IN} ratios in 2 subjects, one (G. E., Table I) belonging to the "non-renal" group, the other (W. T.) manifesting marked hypertension and slight proteinuria. However, in another "normal" (J. J.), the endogenous and exogenous creatinine clearances as determined on 2 successive days did not differ materially.

Effect of Benemid on C_{CR}:C_{IN}:* If a C_{CR}:C_{IN} ratio above 1, particularly as observed following creatinine intake and in patients with renal disease, is due to tubular secretion of creatinine[18, 19, 24] and if C_{IN} equals the glomerular filtration rate, the ratio could conceivably be lowered by suppressing tubular function with Benemid in accordance with similar experiments employing carina-

*Generously supplied by Dr. Gilbert M. Bayne of Sharpe & Dohme, West Point, Pa.

mide.[12, 18, 21, 34] In the "normal" subject mentioned above (J. J.), as well as in 3 patients with renal disease, the possible influence of 1 Gm. of the drug given orally at the end of the third clearance period upon the subsequent creatinine excretion was studied (Table I). Neither in subjects with normal

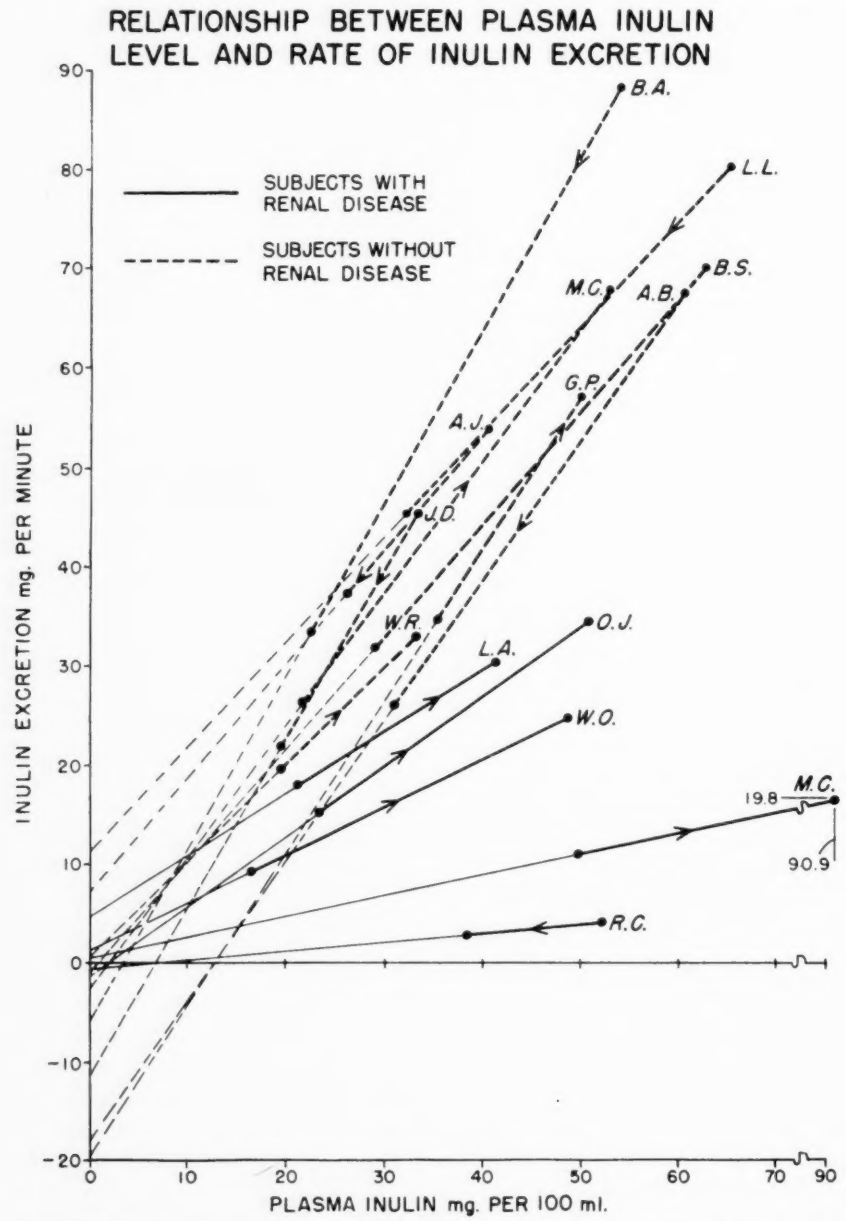

Fig. 3.—Regression lines were drawn from equations shown in Table V, relating the rate of inulin excretion to deliberate changes in plasma inulin concentration. The black dots refer to the highest and lowest plasma inulin observed in each of the 14 subjects so studied. Arrows signify the direction of change in plasma inulin concentration produced.

(J. J., O. P.), nor with pathologically (N. C., W. M.) or artificially (J. J.) raised P_{CR} could such an influence be demonstrated. The ratios of C_{CR}:C_{IN} remained essentially the same although the typical pharmacological effect[35, 36] was evident from the 17 to 300 per cent rise in the simultaneous C_{UA}:C_{IN} ratios.

Influence of P_{IN} Concentration upon C_{IN}: Deliberate changes in P_{IN} had, in 9 "normals" and in 5 patients with renal disease so studied, no consistent influence on C_{IN}, on the ratios of C_{PAH}:C_{IN}, or on the proportionality between P_{IN} and the rate of inulin excretion (Table V, Figs. 3 and 4). The lines relating these two observable parameters (Fig. 3) show a considerable spread, but they intercept the ordinate both above and below the zero point (+11.5 to −19.29 mg. per minute), quite in contrast to Ferguson and co-workers' data[25] which demonstrated such intercepts consistently below zero (−2.91

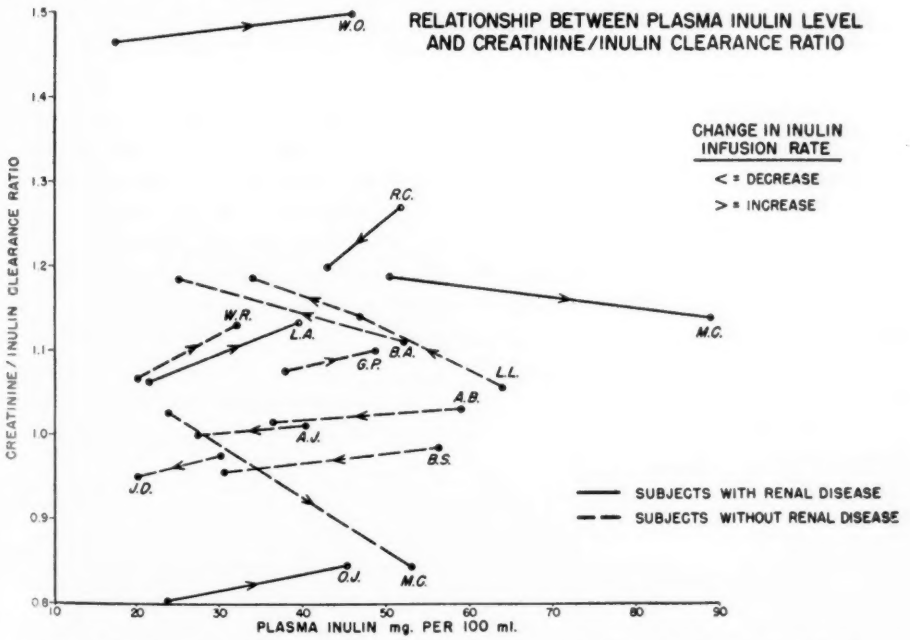

Fig. 4.—Negative slopes, such as L.L., suggest parallelism of changes in C_{IN} with changes in P_{IN} and thus favor dependence of C_{IN} upon P_{IN}. Positive slopes tend to dispute this relationship.

to −14.28 mg. per minute) and therefore suggested to them tubular reabsorption of inulin at this rate. The wide spread of our intercepts may be attributed to the relatively small number of different P_{IN} levels tested (in some instances, smaller than those of Ferguson's) and to the fact that these intercepts actually represent extrapolations. The positive and negative intercepts may therefore be assumed to cancel one another and the true regression line visualized as crossing the ordinate at the zero point. The disparity between those authors' and our findings lacks full explanation since it cannot be entirely due to the large experimental error indicated above; nor can it be readily attributed to differences in analytical methods employed. Whatever variations in C_{IN} occurred in association with P_{IN} changes were largely paralleled by variations

in C_{CR}, which must be independent of the P_{IN} changes (Fig. 4). Obviously, the consistent combination of an increase in P_{IN} with a decrease in the C_{CR}:C_{IN} ratio would be in favor of Ferguson's assertion that C_{IN} is dependent upon the magnitude of P_{IN}. This combination, however, was encountered in 4 subjects only, while in 9 others, including 4 renal patients, the inverse type of relationship was obtained. Again, the negative slopes appear to offset the positive ones resulting in an imaginary horizontal line which would denote independence of C_{IN} from the magnitude of P_{IN}.

DISCUSSION

The experimental error inherent in our clearance measurements, represented by coefficients of variation (Table III), closely agreed with similar estimations published by others (Smith, Goldring and Chasis found 8.9 per cent[37]; Ferguson and associates, 7.0 per cent[25]). These coefficients, though considerable in magnitude, were practically the same for creatinine and inulin clearances. The standard deviations of the clearance ratios within subjects (Table II) were of the same order or even smaller. In other words, however, large the fluctuations of C_{CR} and C_{IN} in a subject may be, they usually occur in the same direction and are of similar magnitude, so that fluctuations of ratios from period to period are proportionately not greater than those of clearances. The inference appears to be that the wide range of ratios encountered even in the "normal" group and the large standard deviations of these ratios between subjects cannot be explained by chance alone.* Hence, one feels inclined to attribute the large discrepancies observed between the two clearances to differences in excretion mechanism for the two substances and the occasional equality of the two clearances to chance. This view is supported by the lack of a marked influence of experimental errors upon the C_{CR}:C_{IN} ratio, as outlined in the preceding section.

While the small number of experiments may not permit one to assign full validity to our statistical conclusions, this weakness seems—at least in part—to be offset by results of similar studies by others which are highly consistent with ours, including a mean ratio of slightly above 1.0 for "normal" subjects.[18, 20, 21] The far wider range and higher mean value of C_{CR}:C_{IN} ratios in subjects with renal disease both in our and in other series[18, 20, 21, 24] appears to emphasize the difference in excretion mechanisms of the two substances under pathologic conditions. Elucidation of this difference might have been aided by more extensive use of renal vein catheterization than has been feasible in this study, particularly in view of the discrepancies between extraction ratios and clearances for creatinine chromogen and other substances noted by Reubi and co-workers.[38] The possible reasons for these discrepancies which those authors advanced cannot be eliminated from considerations of the problem at hand; they include differences in the extraction and blood flow of the separate kidneys, intrarenal conjugation or breakdown of the test substances, removal of part of these substances from the kidney through lymphatic vessels and a consequent by-passing of the renal vein.

*Provided that the clearance ratios do not differ materially on different days in the same subject, a postulate which has been tested and confirmed in 3 subjects only.

The view is widely held that in the normal human kidney creatinine is excreted by glomerular filtration alone, but that tubular secretion of creatinine is added when the plasma creatinine level is increased, either as a result of creatinine administration[3, 8, 18, 34, 39] or of renal disease.[18, 21, 24, 40*] If this is correct, the exogenous creatinine clearance should be expected to be consistently higher than its endogenous counterpart unless and until the increased plasma creatinine concentrations cause tubular saturation and the phenomenon of self-depression comes into play.[18, 39, 41] Further supporting evidence for the above view has been attached to the lowering of the creatinine clearance—in the presence of abnormally high plasma levels—as observed following the administration of phlorizin,[41] diodrast,[39] PAH,[39] or carinamide.[12, 21, 34]

Our data are somewhat at odds with this view: one of the three individuals who ingested creatinine (Table I) showed (at moderately raised plasma levels) no increase in the exogenous over the endogenous creatinine clearance,† a finding which seems to duplicate observations by Hare and associates.[20] Moreover, there appears to be no correlation between the plasma creatinine concentration and the C_{CR}:C_{IN} ratio in patients with renal disease (Fig. 3) in that low concentrations often coincide with high ratios, and vice versa. Benemid, a drug which alters the renal excretion of various substances, such as penicillin, PAH, and uric acid,[35, 36] presumably by influencing tubular transfer processes, was ineffective in lowering the clearance ratios in subjects with either normal or with elevated plasma creatinine (Table I). These observations are analogous to experiences of others with carinamide in acute experiments.[8, 12, 21] Only when given repeatedly over a period of a day or longer preceding the clearance experiment was carinamide intake followed by a reduction in chromogen/inulin[8, 12] or creatinine/inulin[12, 21] clearance ratios.

There is some indication that the *type* of renal lesion may influence the relation of C_{CR} or C_{CHR} to C_{IN}: chronic congestive failure has been shown in one of our cases (O. J.) and in previous studies[8, 9, 13, 18, 24] to be associated with ratios distinctly below unity, suggesting creatinine reabsorption. The case of peritonitis in our series (O. H.), whose renal involvement may be assumed to be primarily circulatory in nature, gave a similar result. On the other hand, cases of nephrosis, such as O. P., seem to tend toward high ratios[8, 24] which presumably denote tubular secretion of creatinine. It is quite conceivable that correlation of these ratios with the exact clinical diagnoses and stages of disease in a larger group of patients, combined, if possible, with determinations of renal extraction ratios[38] and with aspiration biopsies[42] might lead to a better understanding of the excretion mechanisms for creatinine in disease and thus, perhaps, also shed more light on its physiologic aspects.

The apparent independence of the inulin clearance from the plasma concentration of inulin, as suggested by our data (Table V, Figs. 3 and 4), is opposed

*"The most important factor must be the increased plasma concentration of 'creatinine.'"[40]

†Since this subject (J. J.) had the highest endogenous C_{CR}:C_{IN} ratio of all the "normals," one may speculate that his tubules were normally secreting creatinine and did not step up this activity after creatinine ingestion.

to the findings of Ferguson and associates[25] but in keeping with recently published results of similar experiments performed in children with and without renal disease[21] and in normal adults.[43] Thus, the validity of the data of Miller, Alving, and Rubin,[44] attacked by Ferguson, has been vindicated. It must, of course, be borne in mind that all these observations furnish only presumptive evidence in favor of equivalence of the inulin clearance to the glomerular filtration rate. Independence of clearances from plasma concentrations has been reported for urea and for acetone,[45] substances acknowledged to involve tubular transfer processes for excretion.* Moreover, tubular reabsorption of inulin has been plausibly demonstrated to occur in acute toxic nephrosis and allied conditions.[47-49] Consequently, alteration of the usual excretion mechanism for inulin —which presumably involves glomerular filtration only—by pathologic lesions cannot be ruled out in any given case of renal disease, a concept which has been considered by Smith for some time[50] and has recently been accentuated by Oliver.[51] The possibility of pharmacologic interference has been suggested by Reubi and associates.[38]

TABLE V. ANALYSIS OF REGRESSION OF EXCRETION RATE ON PLASMA CONCENTRATION OF INULIN

SUBJECT	EXCRETION RATE OF INULIN MG. PER MIN.	STANDARD DEVIATION OF INTERCEPT	t*	DEGREES OF FREEDOM	P†
	Subjects Without Renal Disease				
J. D.	$-11.30 + 1.70\ P_{IN}$	4.03	−2.83	2	0.11
B. S.	$-0.86 + 1.13\ P_{IN}$	5.06	−0.17	2	0.90
A. J.	$7.16 + 1.15\ P_{IN}$	6.63	1.08	2	0.40
B. A.	$-5.61 + 1.73\ P_{IN}$	2.86	−2.10	3	0.13
L. L.	$11.50 + 1.05\ P_{IN}$	13.69	0.84	4	0.40
A. B.	$-17.81 + 1.40\ P_{IN}$	13.81	−1.29	2	0.30
G. P.	$-19.29 + 1.52\ P_{IN}$	8.65	−2.23	2	0.17
W. C.	$-2.48 + 1.32\ P_{IN}$	2.73	−0.91	4	0.40
W. R.	$0.77 + 0.97\ P_{IN}$	3.12	0.24	4	0.80
	Subjects With Renal Disease				
L. A.	$4.76 + 0.62\ P_{IN}$	2.69	1.77	4	0.15
W. O.	$1.28 + 0.48\ P_{IN}$	0.48	2.64	4	0.07
M. C.	$0.66 + 0.21\ P_{IN}$	0.67	0.99	3	0.40
R. C.	$-0.63 + 0.09\ P_{IN}$	0.73	−0.86	6	0.70
O. J.	$-1.24 + 0.70\ P_{IN}$	2.29	−0.54	3	0.60

Regression equations were calculated by method of least squares.
P_{IN} = Mg. of inulin per 100 ml. of plasma.
*t = Intercept ÷ standard deviation of intercept.
†P = Probability that the observed intercept deviates from the origin by chance alone.

Thus, when viewed within the framework of the prevailing doctrine of renal function,[18] our data suggest that tubular transfer usually plays no role in the excretion of inulin, but that it is operative in the excretion of creatinine in the form of either tubular secretion or reabsorption or of a—presently unpredictable —combination of both, in health as well as in disease. However, if the exchange of fluid and solutes across the glomerular membrane is attributed not to a pressure gradient and consequent filtration but, in accordance with Chinard, to

*On the other hand, a marked dependence of the inulin clearance upon the plasma inulin concentration has recently been noted both in the cat and the dog.[46] By comparison with the creatinine clearance which was not influenced by changes in plasma creatinine concentrations, it was concluded that inulin can be both secreted and reabsorbed by the tubule cells!

the diffusion of molecules caused by gradients in chemical potentials,[52, 53] then such factors as molecular size, electrical potential, and thickness of the membrane must be considered. Since creatinine and inulin differ grossly in the first 2 qualities—creatinine being a much smaller molecule with a positive charge—, one may speculate that the discrepancy in the clearances of these substances may be due to differences in the rate of glomerular diffusion, without necessarily involving tubular processes.

From the practical point of view, the endogenous creatinine clearance appears to be an acceptable clinical substitute for the inulin clearance in subjects without renal disease and also, perhaps, in those with cardiac decompensation. The chromogen clearance falls short of that goal but increasing it by about 25 per cent will approximate it to the creatinine clearance, somewhat in accordance with suggestions by previous authors.[2, 9] In renal disease, these two clearances usually agree more closely while both seem to be poor measures of the glomerular filtration rate—if the latter is represented by the inulin clearance. The continued usefulness of the creatinine clearance as an index of the amount of functioning renal tissue[16, 54] remains, however, unchallenged.

SUMMARY AND CONCLUSIONS

The disparity encountered between simultaneous clearances of endogenous creatinine and inulin indicates that the excretion mechanisms of the two substances are dissimilar even under normal conditions and, especially, in renal disease. The thesis that inulin is excreted by glomerular filtration only receives new support from the data presented which suggest independence of the inulin clearance from the plasma inulin concentration. Consequently, and if the transfer of fluid across the glomerular membrane is conceived as filtration, tubular transfer processes must be invoked for creatinine excretion. The common view favoring secretion as the sole tubular process involved is somewhat weakened by the observations that (a) there was no direct correlation between plasma creatinine concentrations and creatinine/inulin clearance ratios; (b) these ratios were uninfluenced by Benemid ingestion; (c) the ratios may be distinctly below unity in certain (circulatory) renal disorders. Hence other mechanisms, such as tubular reabsorption, must also be assumed to be operative. The clearance ratios encountered in disease appear to be determined by the type of renal lesion, rather than by the plasma creatinine concentration.

If Chinard's concept of glomerular *diffusion* is to be considered, the difference in diffusion constants between creatinine and inulin and alterations in the glomerular membrane may account for the disparity between the two clearances.

The creatinine chromogen clearance is normally about 25 per cent lower—on the average—than the creatinine clearance but approximates the latter in azotemic patients. Within their known limitations, these two clearances appear to be useful clinical substitutes for the inulin clearance only in subjects without renal disease. Their value as indices of over-all renal competency has not been questioned.

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ANTIFUNGAL ACTIVITY OF SOME AROMATIC DIAMIDINES

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THE aromatic diamidines were developed primarily as trypanocidal drugs and, later, were found to be useful in bacterial and neoplastic diseases.[1-3] The antifungal activity of the diamidines was reported first by Elson[4] in 1945 when he presented the results of in vitro tests with propamidine hydrochloride on several fungi pathogenic for man. In the same year, Parsons and Zarafonetis[5] treated two cases of histoplasmosis with stilbamidine. Seabury and Artis[6] in 1946 showed stilbamidine inhibited *Histoplasma capsulatum* in vitro at a level of 10 mg. per cent. Subsequent clinical trials with this compound, however, were not successful.[7]

Schoenbach and associates[8] in 1948 called attention to Elson's report and suggested that the diamidines might prove to be useful chemotherapeutic agents in fungus infections. Colbert and co-workers[9] reported clinical improvement of cutaneous blastomycosis following local therapy with propamidine and Schoenbach and collaborators[10, 11] in 1951-1952 reported clinical improvement in systemic blastomycosis following intravenous therapy with stilbamidine. Since these reports, there have been others describing the use of the diamidines in blastomycosis,[12] actinomycosis,[13] histoplasmosis,[14] cryptococcosis,[15] and chromoblastomycosis.[16]

Clinical use of the diamidines in fungus infections has not been based, for the most part, on a choice of compound determined by its known in vitro activity against a specific fungus. It is the purpose of this report to compare the antifungal activity of four aromatic diamidines by in vitro tests with some of the fungi pathogenic for man.

MATERIALS AND METHODS

Stilbamidine diisethionate, propamidine isethionate, pentamidine and phenamidine* were used in this study since they previously had been shown by Lourie and Yorke[1] to have the best chemotherapeutic index in protozoan infections and have been used clinically in a variety of fungus infections. The compounds were incorporated in warm Sabouraud's glucose agar by means of a Waring Blendor, and plates were poured in concentrations of 10, 5, 2.5, 1 and 0.1 mg. per cent. Duplicate plates containing 20 per cent human serum also were poured.

The antifungal activity of the above compounds was tested against *Candida albicans*, *Cryptococcus neoformans*, and yeast phase cultures of *Blastomyces dermatitidis*, *Blastomyces brasiliensis*, and *Histoplasma capsulatum*. *C. albicans* and *C. neoformans* were incubated at 37° C. on Sabouraud's glucose agar slants for twenty-four and forty-eight hours, respectively. *B. dermatitidis* and *B. brasiliensis* were incubated at 37° C. on beef infusion glucose agar slants for five days, and *H. capsulatum* was incubated at 37° C. on brain heart infusion blood agar slants for seven days.

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All plates were inoculated with 0.1 ml. of a saline suspension of fungus cells obtained by washing the growth from one agar slant, and the inoculum was streaked uniformly over the agar surface by means of a sterile bent glass rod. The plates were read daily for two weeks.

TABLE I. IN VITRO ANTIFUNGAL ACTIVITY OF FOUR AROMATIC DIAMIDINES

		STILBAMIDINE*					PROPAMIDINE					PENTAMIDINE					PHENAMIDINE				
	SERUM	10	5	2.5	1.0	0.1	10	5	2.5	1.0	0.1	10	5	2.5	1.0	0.1	10	5	2.5	1.0	0.1
Cryptococcus neoformans	0	4	4	4	4	4	−	−	+	3	4	−	−	+	+	3	3	4	4	4	4
Cryptococcus neoformans	20%	4	4	4	4	4	−	−	−	±	4	−	−	−	±	4	−	−	−	±	4
Candida albicans	0	+	2	2	2	4†	2	3	3	4	4	2	2	3	4	4	4	4	4	4	4
Candida albicans	20%	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Blastomyces dermatitidis	0	−	−	2	2	3	−	+	3	4	4	3	3	4	4	4	−	+	3	4	4
Blastomyces dermatitidis	20%	−	−	−	+	4	−	±	2	3	4	4	4	4	4	4	−	+	3	4	4
Blastomyces brasiliensis	0	+	+	2	3	4	−	−	−	+	2	−	−	±	+	3	+	+	2	4	4
Blastomyces brasiliensis	20%	+	2	4	4	4	−	−	−	±	4	−	+	2	2	3	+	+	+	3	4
Histoplasma capsulatum‡	20%	−	+	4	4	4	−	−	−	±	4	−	+	2	3	4	−	−	−	±	4

*Concentration in mg. per cent.
†Maximum growth 4; negative growth.
‡Tested only in presence of serum.

RESULTS

The in vitro levels of antifungal activity of the four diamidines tested are presented in Table I. It will be seen in this table that the compounds showed selective activity against the fungi tested. Stilbamidine inhibited *B. dermatitidis* and *H. capsulatum* at 2.5 and 10 mg. per cent, respectively. It did not inhibit *C. neoformans, C. albicans,* or *B. brasiliensis* in the highest concentration tested (10 mg. per cent). Propamidine inhibited *C. neoformans, B. brasiliensis,* and *H. capsulatum* at 2.5 mg. per cent and *B. dermatitidis* at 10 mg. per cent, but was ineffective against *C. albicans.* Pentamidine inhibited *C. neoformans* at 2.5 mg. per cent, *B. brasiliensis* and *H. capsulatum* at 10 mg. per cent, but was ineffective against *C. albicans* and *B. dermatitidis.* Phenamidine inhibited *C. neoformans* and *H. capsulatum* at 2.5 mg. per cent and *B. dermatitidis* at 10 mg. per cent, but was not effective against *C. albicans* or *B. brasiliensis.*

The addition of serum to the medium did not lessen the activity of the compounds. Rather, the antifungal activity of phenamidine against *C. neoformans* was markedly increased in the presence of serum.

DISCUSSION

The results of the in vitro tests referred to above indicate that one diamidine might be better than another as a chemotherapeutic agent for a specific fungus infection. Of the four compounds tested stilbamidine would appear to be the drug of choice in North American blastomycosis, propamidine in cryptococcosis, South American blastomycosis, and histoplasmosis, pentamidine in cryptococcosis, and phenamidine in cryptococcosis and histoplasmosis. It is interesting to note that none of the compounds tested showed activity against *C. albicans.*

SUMMARY

Selective in vitro antifungal activity of four aromatic diamidines against *B. dermatitidis, B. brasiliensis, C. neoformans, H. capsulatum,* and *C. albicans* has been demonstrated. The addition of serum to the medium did not interfere with the activity of the compounds but, rather, enhanced the activity of phenamidine against *C. neoformans.*

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THE VISCOSIMETRIC DETERMINATION OF BLOOD FIBRINOGEN

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THE viscosity of blood and its derivatives have been subject to periodic studies ever since Fahraeus[1] first described the erythrocyte sedimentation rate. Subsequently numerous investigators have compared the sensitivity and reliability of viscosity measurements and the erythrocyte sedimentation rate as indices of disease activity. Whole blood viscosity is largely a reflection of the red blood cell mass, and therefore its usefulness as an index of infection is markedly limited and not comparable to the erythrocyte sedimentation rate. Plasma and serum viscosities are, on the other hand, largely dependent on the protein content and thus are found elevated in disease states just as is the erythrocyte sedimentation rate. Of the proteins fibrinogen is the most potent in causing increased viscosity with the globulins of secondary importance, except in those diseases where the globulin fraction is markedly elevated; for example, multiple myeloma, cirrhosis, etc. The erythrocyte sedimentation rate is affected in a parallel manner by changes in the plasma protein constitution. Fibrinogen has been said to be eight times as effective as equivalent amounts of gamma globulin in causing an increase in erythrocyte sedimentation rate.[2] Increases or decreases of albumin are usually of minor importance in affecting the result of either determination. Reported studies for the most part favor viscosity measurements as being more reliable than the erythrocyte sedimentation rate[3-7] as an indicator of disease activity or in following the course of an illness through remissions and exacerbations. Their relative usefulness has been compared most clearly in tuberculosis and the rheumatoid diseases. These studies of viscosity have been confined largely to plasma measurements. Lawrence,[5] however, has performed some fractional plasma viscosity determinations and noted that there was a correlation between the fibrinogen content of the plasma and the difference in viscosity between plasma and serum. It appeared to the author that careful measurements of plasma and serum viscosities offered possibilities of establishing a simple and accurate means for the quantitative measurement of fibrinogen.

METHODS AND MATERIAL

Twenty to twenty-five cubic centimeters of venous blood is withdrawn without stasis. The fasting state is not necessary if viscosity is the only determination to be made, for solutes other than protein do not seriously affect the viscosity. Half the blood is placed in a serum tube and allowed to clot. The other half is placed in a tube containing the appropriate quantity of dried Heller-Paul potassium and ammonium oxalate.[8] Heparin is also theoretically suitable but has not been used in the present experiments. Other anticoagulants which dilute

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or concentrate the plasma by decreasing or increasing the red blood cell size will lead to errors and should not be used. Each tube is centrifuged for one-half hour at approximately twenty-five hundred revolutions per minute. Particular care must be taken to clear entirely both plasma and serum of red blood cells and to clear the plasma of calcium oxalate crystals, for either will falsely elevate the viscosity. Gross hemolysis must be avoided. Minor degrees of hemolysis are of little importance, however, for the relatively small globin molecule has little effect on viscosity. Exactly five cubic centimeters of serum is introduced into an Ostwald viscosimeter in a constant temperature bath and the time of sedimentation measured to the nearest tenth of a second, in duplicate. Plasma viscosity is similarly measured. The instrument is cleaned and dried with water, alcohol, and acetone between measurements. A single viscosimeter was used throughout for present data, but several instruments can be calibrated against distilled water and used interchangeably. The water bath has been maintained at 37.0° C. for the data reported. However, a more limited number of determinations at 25.0° C. has produced results of essentially the same reliability when applied to a separate calibration curve. In either instance checks to one-tenth of a second are readily obtained for most solutions. Increasing or decreasing the water bath temperature by one-tenth degree gives rise to decreases or increases, respectively, of about one-tenth of a second in viscosity.

The difference in seconds between the plasma and serum is recorded and applied to a calibration curve as the fibrinogen level in milligrams per cent. For purposes of validation and calibration, fibrinogen was also determined chemically in duplicate by the tyrosine method as described by Ratnoff and Menzie.[9] With this method the fibrin clots are collected on pulverized glass following the addition of thrombin to plasma. After digestion the tyrosine content of the fibrin clots is determined colorimetrically and from this the fibrinogen level is calculated. Ratnoff and Menzie found the normal range of fibrinogen to be between 0.164 and 0.415 milligram per cent. (In the present studies fifteen normal subjects were found to have fibrinogen levels between 0.190 and 0.365 milligram per cent.)

Fibrinogen determinations by the method of Ratnoff and Menzie were carried out 100 times on 71 human subjects and compared with the viscosimetric method. The majority of subjects were hospitalized patients with varied illnesses as found on the medical wards of the University Hospital. A disproportionate number of patients had hepatic disease or multiple myeloma because of the author's particular interest in these problems. In several instances serial determinations with both methods were carried out while patients were undergoing therapy.

RESULTS

The data obtained by the viscosimetric method and the method of Ratnoff and Menzie are shown in Fig. 1. Because all of the plasma and sera have been studied with a single viscosimeter, it has been possible for convenience of discussion to record the viscosity values in terms of seconds. They might, however, be equally well recorded in terms of relative viscosity. Included are 16 determinations on 15 healthy men and women, 9 determinations on 6 cases of multiple myeloma, and 75 determinations on 50 patients with varied diseases. The quantity of fibrinogen ranges from a low of 47 milligrams per cent to a high of 1,058 milligrams per cent, or from 0.7 second to 27.2 seconds. The correlation coefficient of the whole group is 0.80. When the values for multiple myeloma are excluded from consideration a correlation coefficient of 0.98 is derived. Fibrinogen in the normals ranges from 0.190 to 0.365 milligram per cent by the method of Ratnoff and Menzie and from 3.0 to 6.6 seconds viscosimetrically. The correlation coefficient for this normal group is 0.88. Viscosimetric values in an additional 9 normal patients not shown in the figure range between 3.1 and 4.7 seconds.

In a total of approximately 250 determinations in a wide variety of diseases the serum viscosity at 37.0° C. has ranged between 52.1 and 131 seconds with the Ostwald viscosimeter used. The time for water under similar circumstances is 39.5 seconds, thus giving a relative viscosity range for serum of from 1.32 to 3.32 in all subjects. This compares with a range of 58.6 to 64.0 seconds in 24 normal subjects or relative viscosities of 1.49 to 1.64.

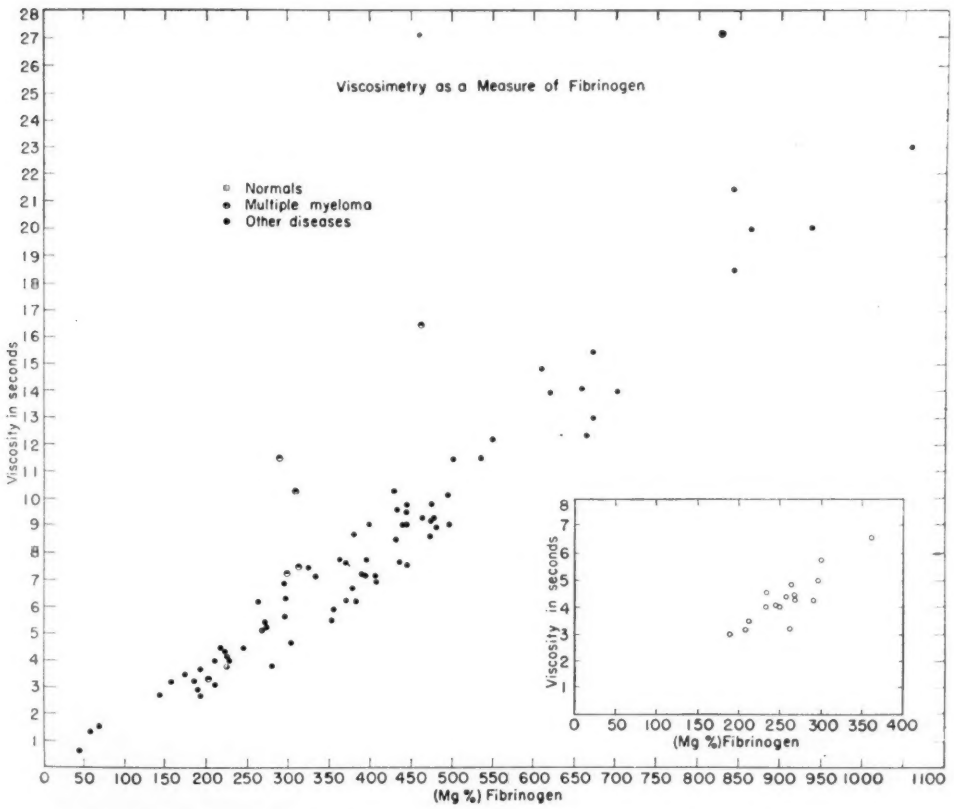

Fig. 1.—The formula for the regression line is: Y = 68 + 41x, where "Y" is the fibrinogen in milligrams per cent as measured by the method of Ratnoff and Menzie, and "x" is the fibrinogen measured viscosimetrically and recorded in seconds.

DISCUSSION

Viscosity is in part a function of the size and degree of hydration of the protein molecule. Probably of more importance is the length and degree of asymmetry of the protein. Whether the fibrinogen in health and disease is identically constituted so far as size, hydration, length, and asymmetry has not been determined. Similarly there may be variations in tyrosine or nitrogen content which would affect any chemical method of determination. With these features remaining in doubt, it would be difficult to state whether viscosimetric or chemical methods more accurately reflect the true fibrinogen content of

plasma. The large discrepancies observed in cases of multiple myeloma are most probably in great measure attributable to viscosity errors. Extreme viscosities of plasma and serum in this disease lead to very slow sedimentation in the Ostwald viscosimeter and thus an additional "jell-like" effect with increased surface tension and consequent false elevation of the true viscosity. Another factor possibly contributing to discrepancies in multiple myeloma is a fibrinogen differing chemically from normal fibrinogen. This is suggested by the known physical and chemical structural variations in the globulins of multiple myeloma. At any rate, it would appear that the viscosimetric method of fibrinogen quantitation should not be relied upon in multiple myeloma in the present state of knowledge.

In the course of the present studies, several instances of cryoglobulinemia have been encountered. The presence of these unusual and peculiar proteins requires particularly careful technique for any method of protein study with special attention to temperature control. Viscosity studies at temperatures ranging from 0° C. to 60° C. have added some interesting data to the characteristics of the cryoglobulins previously described.[10] In two patients with multiple myeloma and clotting abnormalities, large amounts of cryoglobulins have been found which, unlike previously reported cryoglobulins, have not precipitated from solution when cooled, but rather have exhibited a sharp and profound increase in viscosity, amounting to a gel, but without actual loss of solubility. Extensive studies of the clotting mechanism in these two patients by Dr. Paul Frick have also revealed a circulating anticoagulant of unusual nature. These findings will be the subject of a future publication.

With the exception of multiple myeloma the viscosimetric measurement of fibrinogen correlates well with the method described by Ratnoff and Menzie. Inherent in any chemical method of fibrinogen determination is the problem of obtaining a fibrin clot free of absorbed albumin or globulins. This has probably been minimized by collecting the fibrin on pulverized glass. Yet in the present study a significantly large discrepancy was obtained in duplicate determinations by this method. Certainly the intrinsic error of viscosity is less, for the results are readily reproducible in most instances to within one-tenth of a second on duplicate determinations. It also has the advantage of extreme simplicity with little chance for error when the few basic principles noted are adhered to. An additional advantage is that considerable information is supplied in addition to the fibrinogen level, for as noted there is a very narrow normal range of serum viscosity, and the serum viscosity is most often altered in disease as well as the plasma fibrinogen. Rarely have both values been within normal range in a patient with active disease. As might be anticipated, a fair correlation has been found between total serum protein and serum viscosity. Thus many of the cases of hypoproteinemia such as in the nephrotic syndrome have had serum viscosities below the lower limits of normal, whereas virtually all patients with hyperproteinemia have had serum viscosities well above normal.

A major disadvantage of the method as described is the large volume of blood required for the procedure, for serial determinations may necessitate

considerable withdrawal of blood in a seriously ill patient. However, the plasma and serum are in no way altered by the viscosimetric determination and can subsequently be used for other chemical determinations. In the present studies the total and fractional protein, corrected erythrocyte sedimentation in both serum and plasma, and zinc turbidity as well as fibrinogen have been determined on most samples after viscosimetry. Their interrelationships will be the subject of future communications. Preliminary studies have indicated that smaller volume viscosimeters of similar design may give suitably accurate results. Mann[11] has also devised a simple viscosimeter along these lines.

SUMMARY

A simple viscosimetric method of quantitative fibrinogen determination has been described. Its usefulness and limitations have been discussed. The general properties of serum viscosity as a clinical test have been outlined.

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LABORATORY METHODS

COMPARISON OF XENOPUS LAEVIS AND RANA NIGROMACULATA AS TEST ANIMALS FOR THE DETERMINATION OF PREGNANCY

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INTRODUCTION

THE female South African clawed frog, *Xenopus laevis* Daudin is known to be a satisfactory test animal in pregnancy determinations,[1-3] hence the *Xenopus* was selected for use by this laboratory in 1948. Recently several species of male amphibia, studied in various countries, were found to be equally suitable as test animals.[4-7] In view of this fact, and because the relatively expensive *Xenopus* must be imported, it was decided to evaluate the practicability of using a local male frog, *Rana nigromaculata* (Hallowell), for these tests. Since this study was initiated, Farrant and Buckland[20] report that *Rana rugulosa* in Hong Kong and *Bufo melanostictus* in Malaya are sensitive enough for clinical use.

METHODS

During 1950, specimens from 260 patients suspected of normal pregnancy were routinely tested using male *Rana nigromaculata* and female *Xenopus laevis*. Subsequently, follow-up information was obtained from the hospital or dispensary concerned regarding final diagnosis as regards the fact of pregnancy. In addition, 209 patients were tested by the *Rana* alone and these results were also correlated with the clinical findings. The results secured in both series were totaled and reported separately for comparison. When available, the date of the last normal menstrual period was recorded in an attempt to determine the reliability of the test animals in detecting early pregnancies.

The number of false negatives each month in *Rana nigromaculata* was carefully checked for an indication of gonadic inactivity in this frog at different seasons of the year. Similar data on *Xenopus* were also recorded for comparison.

A brief description of the procedure of these tests is outlined below. The method of concentrating and detoxifying the urine is based on the work of Scott.[8]

(a) In tests other than emergency cases, test specific gravity of urine and discard if less than 1.015.

(b) Place urine in a mixing cylinder; 70 ml. are required for concentration in a test on *Xenopus laevis* and 35 ml. for *Rana nigromaculata*.

(c) Add an equal volume of tap water to the urine.

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(d) Adjust the pH to approximately 4 by addition of 20 per cent HCl, drop by drop; determine pH with the nitrazine paper.

(e) For each 35 ml. of urine used, add 2.5 ml. of a 20 per cent suspension of kaolin in water. (Use acid washed kaolin reagent.) Shake.

(f) Allow suspension to settle until kaolin is below the 15 ml. mark.

(g) Decant and discard supernatant.

(h) Transfer kaolin residue to a 15 ml. centrifuge tube and centrifuge for five minutes, at 1,000 to 2,000 rpm.

(i) Decant supernatant liquid. To the residue add 1 ml. N/10 NaOH per 35 ml. of urine used for concentration; stir the mixture thoroughly with a glass rod and centrifuge for five minutes.

(j) Filter; collect filtrate in a clean test tube; add one drop of brom-cresol purple.

(k) Add 5 per cent HCl slowly, stirring after each addition until a pH of approximately 7 is indicated. Use 2 per cent HCl for the last drop. If the end point is passed, add one drop of 1/N NaOH and repeat procedure.

(l) Fill a 2 ml. syringe, equipped with a 23 gauge, ¾ inch needle.

(m) With a fine glass dropper, remove one drop of urine from the cloaca to a clean slide, and examine microscopically to insure the absence of sperm and the possibility of false-positive results.

(n) Insert needle through skin on dorsal side of left leg; avoid injuring large blood vessels in thigh. Push needle under skin into dorsal lymph space which is ¼ inch anterior to the cloacal opening.

(o) Inject the concentrate; one ml. is required for *R. nigromaculata* and 2 ml. for *Xenopus laevis*.

(p) Place test animals in separate jars. Add water to jars containing *Xenopus laevis;* cover jars with towels. Weisman and Coates[9] state that the glare from lights delays or inhibits oviposition by *Xenopus*.

Positive results are indicated by the extrusion of eggs within eight to thirty hours, by the female *Xenopus*, and the release of sperm, within four hours, into the cloaca of the male Rana. A drop of fluid is removed with a capillary pipette from the cloaca of the *Rana* each hour for four hours and examined under the low power of the microscope for the presence of sperm. *Xenopus* can be reused one week after giving a negative reaction, or one month after a positive reaction.[2, 3] In this laboratory the *Rana nigromaculata* were not reused, as the expense of maintaining the colony was greater than the cost of new animals.

RESULTS

A comparison of clinical findings and laboratory results of the parallel tests are summarized in Table I, *A*. Clinical findings showed that of the 260 patients, 136 were normal pregnancies and 124 were not pregnant. *Rana nigromaculata* revealed 85.3 and 91.9 per cent of the positive and negative cases, respectively. *Xenopus laevis* gave correct determinations on 82.4 per cent of the positive and 93.5 per cent of the negative patients. The *Rana* gave correct determinations on 88.5 per cent of the total cases and *Xenopus* on 87.7 per cent. There were 14 inconsistencies in the parallel tests; eight of these were correct by *R. nigromaculata* and 6 by *Xenopus*.

The *Rana* was used alone in 209 tests (Table I, *B*) and gave correct determinations as follows: 88.8 per cent of the positive and 83.3 per cent of the negative cases. The over-all accuracy was 87.1 per cent.

A total of 469 patients were tested by *Rana nigromaculata* in the parallel and single series combined (Table I, *C*); according to the clinical picture 279 were positive and 190 negative for pregnancy. *R. nigromaculata* correctly diag-

TABLE I. COMPARISON OF *Rana nigromaculata* AND *Xenopus laevis* AS TEST ANIMALS FOR PREGNANCY

TEST ANIMAL	TOTAL CASES	EVALUATION OF POSITIVE CASES					EVALUATION OF NEGATIVE CASES					TOTAL CASES CORRECTLY DIAGNOSED	
		NO. POSITIVE BY CLINICAL FINDINGS	LAB. FINDINGS				NO. NEGATIVE BY CLINICAL FINDINGS	LAB. FINDINGS					
			CORRECT POS.		FALSE NEG.			CORRECT NEG.		FALSE POS.			
			NO.	%	NO.	%		NO.	%	NO.	%	NO.	%
(A) Parallel Series Using Both Test Animals													
Xenopus laevis	260	136	112	82.4	24	17.6	124	116	93.5	8	6.5	228	87.7
Rana nigromaculata	260	136	116	85.3	20	14.7	124	114	91.9	10	8.1	230	88.5
*(B) Single Series Using Only R. nigromaculata**													
Rana nigromaculata	209	143	127	88.8	16	11.2	66	55	83.3	11	16.7	182	87.1
(C) Total Tests on R. nigromaculata													
Rana nigromaculata	469	279	243	87.1	36	12.9	190	169	88.9	21	11.1	412	87.8

*This series contains an insufficient number of negative cases to prove statistical significance at a probability of 5 per cent (The Determination of the Statistically Minimum Number of Cases, by R. H. Peckham, Ph.D., Associate Professor of Research Ophthalmology, from Am. J. M. Sc. **224**: 131-134, 1952.)

TABLE II. RELATION OF TEST RESULTS TO DURATION OF PREGNANCY

PERIOD OF AMENORRHEA AT TIME URINE SPECIMEN COLLECTED	*Rana* AND *Xenopus* IN PARALLEL TESTS					TOTAL TESTS BY THE *Rana*		
	DUPLICATE RESULTS			INCONSISTENT RESULTS				
	NO. OF CASES	PER CENT CORRECT POSITIVES	PER CENT FALSE NEGATIVES	NO. CORRECTLY POS. *by Rana* AND FALSELY NEG. BY *Xenopus*	NO. CORRECTLY POS. BY *Xenopus* AND FALSELY NEG. BY *Rana*	NO. OF CASES	PER CENT CORRECT POSITIVES	PER CENT FALSE NEGATIVES
Less than 1 mo.	5	80.0	20.0		1	6	76.7	33.3
1-2 mo.	27	77.8	22.2	1		59	79.7	20.3
2-3 mo.	38	94.7	5.3	1		83	92.8	7.2
3-4 mo.	14	92.9	7.1	1		24	95.8	4.2
4-5 mo.	7	85.7	14.3	2		12	91.7	8.3
5-6 mo.					1	5	60.0	40.0
Total	91	87.9	12.1	5	2	189	87.3	12.7

nosed 87.1 and 88.9 per cent of these patients, respectively. The over-all accuracy was similar to that secured in the single series, namely 87.8 per cent.

Table II summarizes the accuracy of laboratory tests at different periods of pregnancy. The highest efficiency attained by both the *Rana* and *Xenopus* in the parallel tests, namely 94.7 per cent, was on patients in the two to three months' period of amenorrhea. This is shown in Table II.

The highest percentages of false negatives by *R. nigromaculata* occurred in January, August, September, and October, the range being from 12.5 to 13.5 per cent. *Xenopus* gave the highest percentages of false negatives in March, May, August, October, and November; the range being from 12.5 to 19.2 per cent.

DISCUSSION

Statistical Validity.—Differences in results obtained with the two test animals, in duplicate tests, were not statistically significant by the Chi-squared test.[10] Both amphibia were equally accurate in detecting pregnancy.

Period of Pregnancy and Effectiveness of Tests.—The date of the last normal menstrual period was available for approximately half of the cases positive by clinical findings; therefore, the data in Table II may serve as an indication of the trend of the positive results as a whole. Most of the false negatives in the parallel tests occurred in periods of amenorrhea of less than two months and more than four months. The lowest number of false negatives occurred in a period of amenorrhea of two to three months; 94.7 per cent of the patients in this group were correctly diagnosed. These findings coincide with the graph given by Robbins[11] showing the relative amounts of chorionic gonadotropic hormone excreted in the urine during different periods of pregnancy. According to this graph the greatest concentration of hormone in the urine is at seven to eight weeks of gestation, followed by a rapid decline. In the last trimester the hormone is at a low level.

An evaluation of the total tests with the *Rana* on cases positive by clinical findings showed results similar to those in the parallel tests (Table II).

Analysis of False Negative and False Positive Results.—The male *Rana pipiens* was reported 99.5 per cent accurate with no false positives, in a study by Soucy[6] and 96 per cent accurate with the occurrence of only one false positive in an evaluation by Robbins and co-workers.[5] Dittebrandt[3] found *Xenopus* 98.8 per cent accurate in an evaluation of 567 tests; Robbins and associates[2] reported an accuracy of 96 per cent in a study of 100 tests. There is no evidence in the available literature of false positives by *Xenopus*.

Our findings with *R. nigromaculata* and *X. laevis* in contrast to the findings mentioned above are approximately 10 per cent lower. Errors in reporting the clinical findings accurately may account for several of the incorrect determinations. Follow-up data are notoriously subject to error by selection, and with a transient population of military dependents, it is all too easy to realize the difficulty in any one installation continuing observation on many of the patients throughout a gestational period. The following additional factors also must be considered:

(a) *Low Hormone Titer of Urines from Early or Late Pregnancy.*—This situation is normally encountered in routine pregnancy tests and results in an unjust evaluation of the effectiveness of the test animals. That some of the false negatives in this study were due to this factor is suggested by the fact that the date of the last normal menstrual period was not listed for 11 of the 36 false negatives by *R. nigromaculata* (Table 1, *C*). Possibly these 11 patients were tested before a menstrual period was missed, or, less likely, were in late pregnancy. Cases in which the hormone titer was low throughout early pregnancy (i.e., first trimester) must also be considered in this category.

(b) *Efficacy of Method of Concentrating Urine.*—Reinhart and associates[12] found that *Rana pipiens* gave an over-all accuracy of 91.4 per cent on urines concentrated by a kaolin method similar to Scott's[8] and an accuracy of 99 per cent on urines concentrated by a cold acetone procedure. In our study *Xenopus laevis* and *Rana nigromaculata* yielded an efficiency of 87.7 and 88.5 per cent, respectively (Table 1, *A*). The lower results by the kaolin method on three different frogs is of interest. An equivalent of only 5 to 10 ml. of urine are needed in the cold acetone procedure in contrast to 35 to 45 ml. of urine equivalent in the kaolin method; this indicates that the former method removes gonadotropic hormones more effectively and suggests that a higher efficiency can be attained, by this method on urines of low hormone titer.

(c) *Deterioration of Hormone in Unpreserved Urines.*—Some unpreserved urines were in transit for several days, and sufficient deterioration of hormone may have occurred to produce a negative result. Two urines, however, which were exposed to room temperature for nine days, were still positive by the *Rana.* Deterioration in these specimens did not deplete the hormone content below a reactive level. Dittebrandt[3] states that chorionic gonadotropins are not stable in unpreserved urines. Robbins and collaborators[5] also mention that deterioration of hormones contributed to some of the false negative results in his study on *Rana pipiens.*

(d) *Seasonal Gonadic Inactivity of Test Animal.*—Soucy[12] and Holyoke and colleagues[14] observed that the increase in false negative and weak positive reactions by *Rana pipiens* during the summer months was caused by an ebb in spermatogenesis in the frog at this season.

Data were collected each month to determine whether *Rana nigromaculata* was also subject to cyclic spermatogenesis. The highest number of false negatives occurred in August, September, and October, followed by a drop during the next two months and a rise in January. These data are not clear, and the subject of a seasonal inactivity or dormancy of the *Rana* testes, as well as the life history of the frog, requires further study. Ting and Boring[15] studied the seasonal reproductive cycle of mature *Rana nigromaculata* in the west Peking area of China. They observed that the mating season extended through April and May, that spermatogenesis began during this period, and continued with increasing vigor until late October at which time practically all the germ cells had developed into mature sperm. Spermatogenesis ceases in late fall. Throughout the year mature sperm are present in the testes, however, in May, June, and

July they are greatly outnumbered by germ cells in the process of maturation. Any false negatives due to a paucity of functional sperm in the testes, as assumed above, should have occurred during these months instead of late summer and early fall unless the spermatogenic cycle of the Japanese *Rana* is later. Cei,[16] Witschi,[17] and Galgano,[18] studying European frogs, noted that a species of frog may have a different spermatogenic cycle depending on geographic locations. Matsuda[19] states that the mating season of the Japanese *Rana nigromaculata* begins in May and extends to the end of July; if this is true then the spermatogenic cycle would be later in frogs that had mated late in the season.

There is a possibility that in some frogs, after the breeding season, the testes are exhausted or contain few functional sperm. This is indicated by the fact that during late summer and early fall some of the larger, apparently older frogs are slow to react or produce weakly positive or false negative reactions, whereas the same amount of urine concentrate injected into another *R. nigromaculata* of the same size yielded a normal positive reaction. Another sign which may mean decreasing gonadic activity is a slight reduction in the size of the nuptial pad, or thickening of the skin at the inside of the base of the inner finger of the front feet. During the breeding season these pads are well developed. Although only a few frogs were dissected, in no case was the distal portion of the ureters (so-called seminal vesicle) enlarged during the fall months; however, functional sperm were observed in the macerated testes. The number of false negatives by this frog that could be attributed to gonadic inactivity is less than that reported for *Rana pipiens*. During the late summer it was observed that frogs of medium size, i.e., specimens in the second year of growth (50 mm.), gave stronger and faster positive reactions. Subsequently in 1951 this size frog was used, when available, for the tests in late summer.

It is known that *Xenopus laevis* ovulates throughout the year and, since it gave about as many false negatives as *Rana nigromaculata* during a time when the latter appeared less responsive, it is believed that the majority of the false negatives by the two-test animals was due to urines of low hormone titer.

False positives are difficult to explain especially for *Xenopus* which is not known to yield this type of result. In the case of *Rana nigromaculata* several frogs during January, February, and March, 1951, were found to contain a few cloacal sperm prior to a test. This was not noted in 1949 and 1950, although the animals were screened. Usually these animals appeared sickly, dying shortly after the examination; however, some lived several days. Since each animal was carefully screened before use in a test, the possibility of false positives by this factor is considered negligible, but cannot be ignored completely, especially in nonnormal pregnancies. It is thought that the false positives are due to errors in reporting clinical results, for example some of the patients, correctly diagnosed as positive by the *Rana* or *Xenopus*, may have later had a miscarriage simulating an irregular or an unusual menstruation which was regarded by the patient as a delayed menstrual period. Further medical attention may have resulted in only a brief entry of "not pregnant" in the history; in such an instance the laboratory test would be labeled a false-positive.

Biology of Rana nigromaculata.—The adult male as distinguished from the female is smaller, its weight being about 30 grams and length (snout-vent) 65 mm.; it is further differentiated by a broader head, rounder snout, longer and stronger forelegs, large vocal sacs located near the edge of the jaw, posterior to the eye, and the presence of pads, or greyish black thickenings of the skin at the base of the inner finger of the foreleg. The latter are more highly developed during the breeding season.

The coloration of both sexes is similar. The dorsolateral surface of the male head and trunk varies from green to a greyish brown. Dorsally there are two parallel stripes of elevated integument of a golden-brown color; between these stripes there are usually several pairs of black spots likewise elevated and surrounded by a brown coloration which often fills the space between the spots. In some specimens there may be a green or white stripe between the golden stripes. A row of black spots near the ventral edge of the brown stripe is also characteristic of this species. The dorsal surface of the limbs are of a brown color interspersed with black spots.

According to Matsuda,[19] the *Rana* enter hibernation during the first frost and remain inactive for five to seven months, depending on the locality. Upon emerging in the spring they shed their outer skin and grow rapidly. Each female lays about 4,000 eggs during one amplexus. Ting and Boring[15] describe the growth of *R. nigromaculata* in West Peking, China, as follows: Frogs hatched in the spring are about six months old in October and are fully mature two years later (i.e.) two and one-half years after hatching. At six months of age, the frogs do not possess nuptial pads or vocal sacs, testes are very small and sexes indistinguishable externally. Frogs one and one-half years old measure 45 to 50 mm. in length (snout-vent), nuptial pads and small vocal sacs are present, and the testes contain active sperm.

CONCLUSION

The results of the study using *Rana nigromaculata* and *Xanapus laevis* in parallel tests on 260 patients, 136 of which were positive and 124 negative for pregnancy, show that these amphibia are equally accurate as test animals. Differences in results were of no statistical significance.

Rana nigromaculata used alone in tests for pregnancy on 209 patients gave results that were not statistically different from those secured by this frog in the parallel tests with *Xenopus laevis* on 260 patients.

Rana nigromaculata can be used where available as a test animal for the detection of normal pregnancies, since it gives as good results as those obtained with *Xenopus laevis.*

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AN APPARATUS EMPLOYED IN PLATE COMPLEMENT-FIXATION TESTS

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IN 1949, Fulton and Dumbell[1] described the plate complement-fixation test, a drop method, for the serologic comparison of strains of influenza viruses. Later Svedmyr, Enders, and Holloway[2, 3] employed the plate complement-fixation test with Type 1 (Brunhilde), Type 2 (Lansing) and Type 3 (Leon) strains of poliomyelitis viruses propagated in tissue culture. In this laboratory we have used the drop method successfully for the assay of antiserums obtained from monkeys receiving intramuscular injections with each of the three known types of poliomyelitis viruses. The complement-fixing antigens employed in our work were prepared according to the method of Svedmyr and associates.[2]

The apparatus as described by Fulton and Dumbell,[1] although adequate for research purposes, lacked certain features desirable for routine tests. Consequently an apparatus was designed which not only maintains the necessary conditions of temperature and humidity, but also saves time and space.

The apparatus shown in Fig. 1 is one of two identical units utilized in our laboratory for performance of the plate complement-fixation test. The cabinets (height: 9¾ inches; width and depth: 12¼ inches) are constructed with ⅜ inch Plexiglass II having a thermal service temperature of 180° F. Each is designed to be airtight in order to maintain a water-saturated atmosphere. In addition to airtight bonded joints, the edges of the opening are rimmed with Newcork strips. Two tension locks insure ease of opening and closing of the flap door which is secured to the box with brass hinges.

Each cabinet accommodates 5 Plexiglass plates which slide freely onto a ¼ inch track. The cabinet is designed so that the distance between the individual plates as well as the place between the first plate and the top of the box is 1 inch. Each plate (12 inches square and ¼ inch thick) is engraved with one hundred 1 inch squares, with numbering and lettering as shown in Fig. 1. Each plate is the equivalent of one hundred serologic tubes which might be employed in a corresponding test.

In order to prevent drying of the antigen-antibody system, cotton moistened with water is layered in the bottom of the airtight box. The humidity over a twenty-four hour period remains relatively constant. One box is kept in an ordinary (8 cubic foot) sized refrigerator at 4° C., and the other is kept in a small incubator at 37° C.

The various serologic methods used in the plate complement-fixation test have been published previously.[1, 2, 4] The desired quantity of each reagent may be readily obtained by using blunt 19-gauge needles on tuberculin syringes

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calibrated to deliver 0.2 ml. (±0.04 ml.) per drop.[2] Titrations of hemolytic system and complement are easily and quickly done. Endpoints in reading the preliminary and final test results are usually quite sharp. Reading of tests has been facilitated by resting the Plexiglass plate on an illuminated frosted glass viewing box.

The use of the drop method has a distinct advantage in sparing antigens which are difficult and expensive to prepare, such as is momentarily the situation with regard to poliomyelitis virus antigens. It is also of potential value, and would appear well suited for use in serologic surveys, particularly during epidemic outbreaks of infectious diseases, or wherever a large number of tests must be done in a brief period of time.

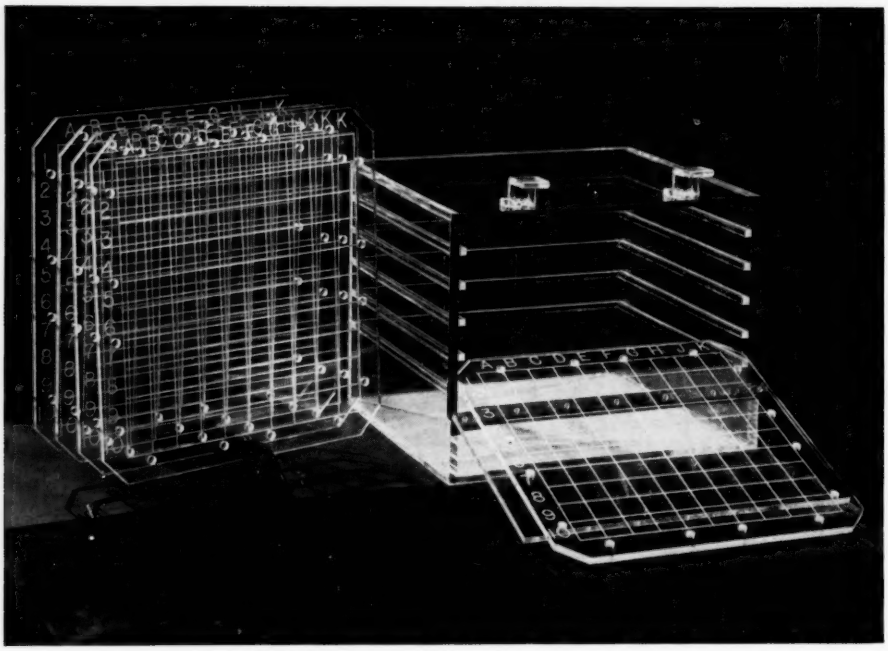

Fig. 1.—Apparatus designed for use in plate complement-fixation test.

SUMMARY

An apparatus designed to facilitate the performance of the plate complement-fixation test is described.

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A KNOWN ENZYME CONCENTRATION AS A CONTROL IN THE ALKALINE PHOSPHATASE TEST

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A WIDELY used method for the determination of serum alkaline phosphatase activity[1] generally applied in the field of clinical chemistry makes use of an inorganic phosphate solution as a standard for the estimation of the phosphorus ion liberated by the enzyme. Another technique using disodium phenylphosphate as a substrate and phenol as the standard to measure the liberated product[2] is also widely used. From the definition that a unit of phosphatase activity is "equivalent to the liberation of 1 mg. of the phosphorus ion or of phenol during the first hour's incubation under the circumstances described," it is obvious that the standard solution is grossly inadequate as a control of the efficiency of the test performance. These standards suffer from the drawback of by-passing both the incubation period and the enzymatic hydrolysis to which the unknown samples are subjected. The purpose of this communication is to propose the introduction of an actual enzyme-containing serum of known concentrations as a more satisfactory control of serum alkaline phosphatase activity testing. This control would be subjected to all the reagents and manipulations applied to the unknown samples during the performance of the test. It is felt that this type of control would provide a greater indication of the efficiency of the test procedure.

The suggestion is based on published evidence[3-5] and on our own experimentation indicating that the alkaline phosphatase activity of human blood serum remains relatively constant for months if the sample is properly stored under deep freeze conditions. In 1951 Ruppert[3] published data to show that while 50 per cent of the alkaline phosphatase activity was lost if the sample was kept at 37° C. for five days, the same sample lost only about 5 per cent if kept at 4° C. Ainslie, Stumpf, and Francis,[4] in a study of serum alkaline phosphatase activity in poliomyelitis, found that there was no difference in the activity between aliquots of sera frozen for various intervals. They studied samples frozen at minus zero degrees centigrade from one day to five months. Recently Davison[5] published an observation that the serum containing acid phosphatase could be stored similarly without appreciable change in activity.

METHODS AND RESULTS

Table I is representative of the results we obtained in testing different serum samples of varying concentration frozen for periods up to seven months. Sixty days was arbitrarily chosen as a desirable storage period. The specimen

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was allowed to clot and the clear serum removed. An alkaline phosphatase determination was performed on a portion of the sample immediately, and aliquots of the rest of the specimen were frozen. For freezing the sample was placed in a small test tube to minimize the air space above the fluid. The tube was corked, sealed with cellulose tape, and the top of the tube dipped in melted paraffin. The tubes were then placed in plastic freezer bags and stored in a deepfreeze unit below zero degrees centigrade. At the varying time intervals indicated in Table I a tube was removed from the deepfreeze, thawed by hand warming, and tested for the alkaline phosphatase activity using a modified Bodansky procedure. The results are in Bodansky units.

TABLE I. STABILITY OF SERUM ALKALINE PHOSPHATASE ENZYME STORED BELOW ZERO DEGREES CENTIGRADE

SAMPLE	FRESH	30 DAYS	60 DAYS	210 DAYS
No. 1	28.5	29.0	25.5	26
No. 2	7.6	8.0	8.5	
No. 3	13.2	14.1	17.0	
No. 4	16.2	18.0	16.9	

The above table substantiates the published observations that the alkaline phosphatase activity of human serum remains relatively constant for long periods if the samples are properly stored. The minor variations in analytical results are apparently within the range of the reduplication of the procedure. Other workers have noted the same plus or minus 10 per cent limit in the accuracy of the enzymatic procedures. Davison[5] recorded 220 King-Armstrong units on the sixth day, 195 units on the sixteenth day, and a return to 210 units when the same sample was tested on the one hundred twelfth day.

Once a sample has been tested and the concentration established aliquots from storage may be used as a known standard enzyme control over the test reagent and technique. As a further check recovery experiments as illustrated in Table II can be performed.

TABLE II. RECOVERY OF ALKALINE PHOSPHATASE ENZYME WHEN ADDED TO UNKNOWN FRESH SERUM SAMPLES

FROZEN SERUM NO. 4	FRESH SERUM	OBSERVED ACTIVITY	EXPECTED ACTIVITY	PER CENT RECOVERY
0.5 c.c.	0.0 c.c.	18.0	16.0	110
0.2 c.c.	0.3 c.c.	11.0	11.1	100
0.0 c.c.	0.5 c.c.	6.5	—	—

A purified dried crystalline preparation of the enzyme that would remain stable at room temperature is desirable. The difficulties involved in making such a preparation and the present impossibility of producing one has been well elaborated by Mathies.[6] He found that his dried crystalline preparation, even though frozen, did not keep as well as an aqueous solution of the crystals stored under the same conditions. It would seem that at the present time the suggested method of deep freezing a calibrated serum sample is the only simple method available for keeping the alkaline phosphatase enzyme.

SUMMARY

Repeated observations and recovery experiments using serum samples as controls for the alkaline phosphatase activity test indicate that the original enzyme activity is retained for a long period of time if properly frozen. The use of the calibrated frozen serum is proposed as a better control for serum alkaline phosphatase determinations.

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USE OF 1,2-DICHLOROETHANE IN THE CARR-PRICE ANTIMONY TRICHLORIDE REAGENT FOR THE DETERMINATION OF VITAMIN A

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THE Carr-Price[1] colorimetric method for the determination of vitamin A has been used extensively in research laboratories. However, its use in routine clinical laboratories has been limited because of the difficulty of preparing the Carr-Price reagent, which is a solution of antimony trichloride in anhydrous, alcohol-free chloroform. When vitamin A is treated with the reagent, a bright blue color is produced.

As most brands of chloroform contain ethyl alcohol as a preservative, the first step is to remove the alcohol, since its presence is likely to produce a cloudy reagent, which is unfit for use. The chloroform is therefore washed with water four successive times by thoroughly shaking it with water in a separatory funnel each time. The washed chloroform is dried over anhydrous calcium chloride for a few days with occasional shaking. The chloroform is then poured into a flask containing fresh anhydrous calcium chloride, and the chloroform is distilled with the aid of an efficient fractionating column such as that of Clarke and Rahrs.

The Carr-Price reagent is prepared by dissolving 22 Gm. of Baker's C.P. antimony trichloride in 100 ml. of the purified chloroform and filtering the solution through a rapid filter paper (Whatman No. 12) to remove any particles present. The reagent is stable for a month or two and then commences to deteriorate due to the decomposition of the purified chloroform.

MATERIALS AND METHODS

We have recently found that 1,2-dichloroethane (ethylene chloride) may be used in place of chloroform in preparing the Carr-Price reagent. The 1,2-dichloroethane is obtained from Eastman Kodak Company, Rochester, N. Y. It is listed as Eastman Organic Chemical No. 132. It is used without any purification. Thirty grams of Baker's C.P. antimony trichloride dissolve readily in 100 ml. of the dichloroethane, the solution is filtered, and is ready for use. Thus far, the reagent has proved quite stable. If too dilute solutions of antimony trichloride in ethylene chloride are used, the blue color obtained when vitamin A is treated with the solutions tends to be more transient. The practical grade of the dichloroethane, P No. 132, does not give as satisfactory a reagent.

1,2-Dichloroethane is well adapted for use in the clinical laboratory. For example, it is the solvent used in Coburn's[2] method for determining the plasma level of salicylic acid. However, the same precautions should be employed in handling it as in handling chloroform. Prolonged breathing of its vapor should be avoided. If it is necessary to pipette the dichloroethane, it is best to use a rubber tube on top of the pipette instead of placing the pipette directly in the mouth. Used solutions of the dichloroethane should be promptly washed down the sink with plenty of water.

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In our laboratory, the macro unit of the Evelyn photoelectric colorimeter, with filter 620, is used for the determination of vitamin A. The "blank" for obtaining the "center setting" for the vitamin A determination is prepared by placing 0.5 ml. of the 1,2-dichloroethane in the bottom of a macro tube and adding 10 ml. of the dichloroethane antimony trichloride reagent (measured in a small cylinder).

The solutions and extracts of vitamin A to be analyzed are usually made with petroleum ether, though other suitable solvents could be used. A few crystals of hydroquinone are added to the solutions to prevent oxidation of the vitamin. A measured amount of the vitamin A solution to be analyzed is pipetted into the bottom of a macro tube which is placed in a beaker of water warmed to about 40° to 45° C. The solvent is completely evaporated by means of a gentle current of air introduced into the tube. The residue in the bottom of the tube is dissolved in 0.5 ml. of the dichloroethane, the "center setting" of the colorimeter adjusted if necessary, and the tube placed in the holder. Ten

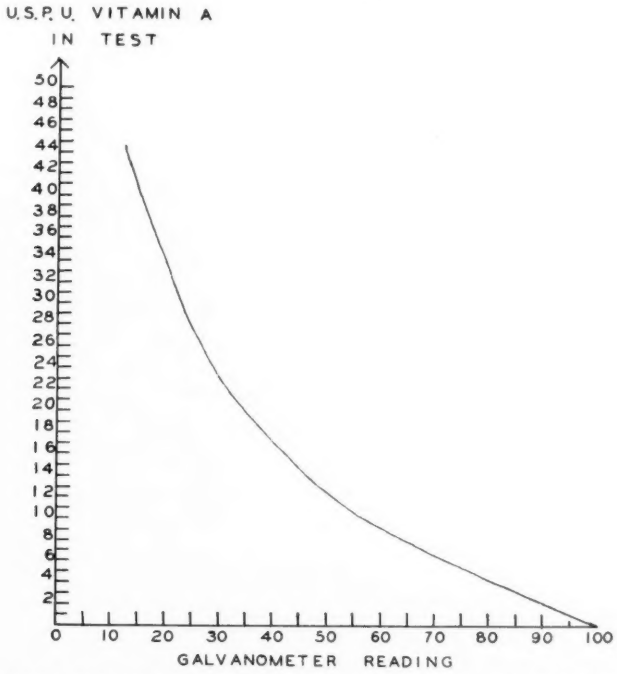

Fig. 1.—Curve for the determination of vitamin A with the macro unit of the Evelyn photoelectric colorimeter. The "blank tube" contains 0.5 ml. of 1,2-dichloroethane and 10 ml. of the 1,2-dichloroethane antimony trichloride reagent. In a second macro tube the dry residue containing the vitamin A is dissolved in 0.5 ml. of the dichloroethane, the tube is inserted into the holder, 10 ml. of the antimony trichloride reagent added and the galvanometer reading recorded. The number of U.S.P.U. of vitamin A in the tube is then obtained from the curve.

milliliters of the dichloroethane antimony trichloride reagent are added immediately, and the indicator of the galvanometer will pause for a few seconds at the correct reading before the bright blue color developed begins to fade.

The number of U.S.P.U. (United States Pharmacopoeia Units) of vitamin A in the petroleum ether aliquot analyzed may then be determined from the calibration curve shown in Fig. 1. This method for determining vitamin A can be adapted readily for use with other types of colorimeters. Vitamin A acetate and vitamin A alcohol suitable for preparing calibration curves may be obtained from Eastman Kodak Company.

When the same amounts of vitamin A are treated with the dichloroethane antimony trichloride reagent, and the chloroform antimony trichloride reagent of Carr-Price, the intensity of the blue color developed is the same for the two reagents. Both reagents also give a green color with vitamin A aldehyde.

Inasmuch as the carotinoid pigments, carotene and xanthophyll, are usually present in petroleum ether extracts of human blood and tissues, and since they also give a blue color with the antimony trichloride reagent, corrections can be made for their presence. The amounts of carotene and xanthophyll in the solutions are determined by the method of Clausen and McCoord.[3] When 100 gammas of carotene are treated with the antimony trichloride reagent, they give a blue color equivalent to 17 U.S.P.U. of vitamin A, and 100 gammas of xanthophyll, a blue color equivalent to 22 U.S.P.U. of vitamin A.

RESULTS AND DISCUSSION

Many physicians have expressed the wish that vitamin A determinations could be done in the clinical laboratories with which they are associated. However, in most instances, the preparation of the chloroform for the Carr-Price reagent has proved too difficult for young, inexperienced technicians, or the apparatus required for its preparation has not been available. The substitution of 1,2-dichloroethane, which needs no purification, for the purified chloroform makes the preparation of the antimony trichloride reagent a simple procedure, and thus the determination of vitamin A more available to all.

The ability to determine vitamin A in the blood and various other body fluids and tissues is a most desirable addition to the armamentarium of a clinical laboratory. A knowledge of the concentration of vitamin A in the blood serum may confirm the diagnoses of vitamin A deficiency, hypervitaminosis A, Bright's disease, hypothyroidism, etc.[4] The vitamin A absorption test, which is based on the increase in the concentration of vitamin A in the serum after ingestion of a test dose of vitamin A alcohol, aldehyde, or ester, has proved most useful in making differential diagnoses. The amounts of these various vitamin A preparations which the patients are able to absorb are characteristic for such diseases as celiac disease, fibrocystic disease of the pancreas, giardiasis, intestinal tuberculosis, catarrhal jaundice in its various stages, etc.[5]

SUMMARY

1,2-Dichloroethane may be used in place of chloroform in the preparation of the antimony trichloride reagent of Carr-Price, which is used for the colorimetric determination of vitamin A. The 1,2-dichloroethane No. 132 which is obtained from Eastman Kodak Company, Rochester, N. Y., needs no purification before being used in the reagent.

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A SATISFACTORY CONSTANT INJECTION APPARATUS

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THE apparatus depicted in Fig. 1 is meeting all needs in our laboratory for constant injection at the hourly rates of 0.33, 1.0, 2.0, 3.0, 4.0, and 5.0 ml. We have twelve of the assemblies in operation, having built them in our own shop at very slight cost.

The force propelling the plunger downward in the syringe is the constant pull of the rubber band. The rate of the descent is determined by the circumference of the pulley around which the string is wrapped, this pulley being attached by set-screw to the shaft of a synchronous motor (Hansen Manufacturing Co., Princeton, Ind.) making one revolution in twenty-four hours. The extent of the downward excursion of the plunger in twenty-four hours determines the amount of string that must be unwound from the pulley in that time; i.e., determines the circumference of the pulley. Knowing the circumference we can derive the diameter, lay out the circle on a sheet of plastic material, and cut out the pulley. The following are the calculations for deriving the diameters of a typical set of pulleys.

The distance between zero and 24 ml. on a certain 30 ml. syringe is $2\frac{17}{32}$ inches, or 2.5312 inches in decimal equivalents. This syringe will therefore deliver 24 ml. in twenty-four hours (or 1 ml. per hour) provided 2.5312 inches of string are unwound from its pulley in that period. So the circumference of the pulley, which is to make one complete revolution in twenty-four hours, must be 2.5312 inches. This figure, divided by 3.1416 (pi), reveals that 0.805 inch is the diameter to be used in making the pulley to deliver 1 ml. per hour. The diameter of the 2 ml. pulley must be twice 0.805, or 1.61 inches; of the 3 ml. pulley, 2.415 inches; of the 4 ml., 3.22 inches; and of the 5 ml., 4.025 inches.

To determine the diameter of a pulley to deliver 0.33 ml. hourly from one of our 10 ml. syringes, we multiplied 0.33 ml. by 24, which yielded 8 ml. as the total amount of fluid that would be delivered from the 10 ml. syringe in the twenty-four hours consumed in one revolution of our synchronous motor. The distance between zero and 8 ml. on this syringe barrel being $1\frac{25}{32}$ (1.7812) inches, we divided this by 3.1416 to obtain 0.566 inch as the diameter required to deliver 0.33 ml. per hour.

When using the syringes ordinarily available in laboratories it is necessary to calculate the pulley sizes for each individual syringe. This is obviated for any series of syringes of equal capacity by using Ideal interchangeable plunger syringes, which are no more costly than the others. In our assemblies the six pulleys, providing the hourly range of 0.33 to 5.0 ml., are housed on a pin from

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which the one desired can be selected easily. The assembly also carries its own pair of syringes, one of 30 and one of 10 ml., and a small screw driver for use on the pulley set-screw.

To fill the syringe and tubing we remove the two rubber bands that hold the former in its rack and detach the latter. Fluid is then drawn up into the syringe somewhat above the zero mark, the tube is reattached and its air expelled in the usual manner, and the filled syringe and tubing are put back in place. The string from the pulley is attached to the top of the syringe and the

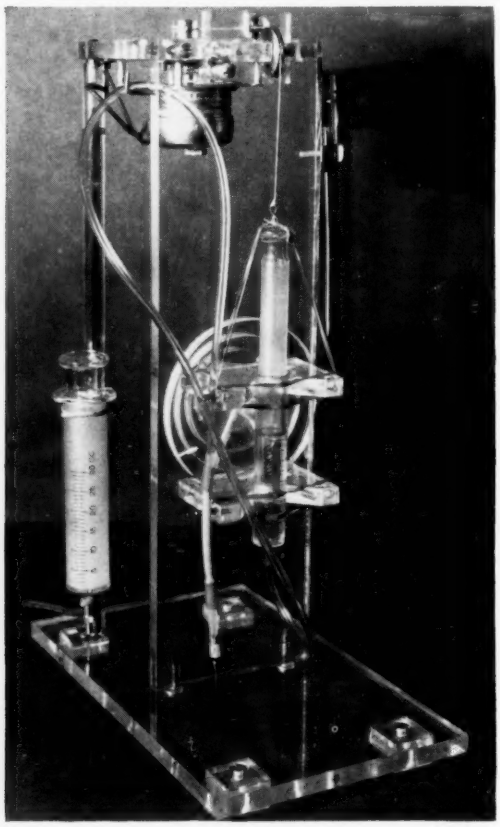

Fig. 1.

set-screw tightened to hold it taut. Then the rubber band that will pull against the motor is attached below. By use of the key that is a part of the synchronous motor unit, the forward end of the plunger is brought precisely to the zero mark on the syringe barrel. The needle is then introduced into the vessel or other structure that is to be injected, and the current is turned on. The delivery of the solution thereafter is automatic.